약학박사학위논문

Surface Camouflaged and Genetically Engineered Pancreatic Islet Transplantation for the Treatment of Diabetes Mellitus

당뇨병 치료를 위한 표면 개질 및 유전자 조작 췌장소도의 이식

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제약학과 물리약학전공

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지도 교수 변 영 로

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위 원 _____ (인)
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Abstracts

Surface Camouflaged and Genetically Engineered Pancreatic Islet Transplantation for the Treatment of Diabetes Mellitus

Jee-Heon Jeong

Department of Pharmaceutical Sciences

College of Pharmacy

Seoul National University

Exogenous insulin therapy is currently used for the treatment of type 1 diabetes mellitus. However, insulin therapy induces unwanted complications such as hypoglycemia, allergy reaction, and so on. Recently, pancreatic islet transplantation is a promising strategy to treat type 1 diabetes, as it can achieve strict regulation of blood glucose level. However, the limited availability of cadaveric pancreas, donor shortage, immune rejection and autoimmunity are the major hurdles for successful islet transplantation. Especially, transplanted islets cell antigens are recognized by antibodies from recipients and it is the most difficult barrier to firm faces for successful islet

transplantation. To overcome this problem, The 'Edmonton protocol' that is cocktailed immunosuppressive drug therapy has been used from 2000. Although known as one of the best accepted protocols outlined for islet transplantation in clinic to date, has shown lower than 10% only of its recipients achieving insulin independence ratio after 5 years of transplantation. Thus, a new immunoprotective remedy has been in needs to inhibit host immune reaction.

In this study, islet donor shortage is one hurdles revealed from the past islet transplantation studies. At least 2-3 cadaveric pancreases were needed for treatment of one diabetic patients using islet transplantation. Recently, stem cell therapy and porcine islets transplantation have been accessed to solve this problem. However, it takes much time to apply these technologies to clinical study. Therefore, this study assessed that the newly designed exendin-4 (Ex-4) gene with highly releasable characteristics could enhance the beta cell function, thereby attenuating the essential islet mass required to cure diabetes. We constructed a lentiviral vector system encoding for a highly releasable secretion signal peptide, the peptide linked Ex-4 (SP-Ex-4) gene. After the transduction of lentivirus encoding for SP-Ex-4 (LV-SP-Ex-4) gene into the islets, the rate of insulin secretion was three-fold increased. In addition, 50 islets expressing Ex-4 were transplanted to cure the diabetic nude mice, whereas at least 150 untransduced islets had to be transplanted to cure the diabetic nude mice. When the transplanted islets were transplanted into diabetic immunocompetent mice, the survival time of islets was 18.0 ± 4.9 days; however, when the untransduced islets were transplanted, they were

rejected within 10.0 ± 0.6 days. Therefore, the highly releasable Ex-4 could enhance the beta cell function with slightly enhanced viability of transplanted islets, presenting as a potential technology for overcoming islet shortage.

Host immune reaction is another blockade for successful islet transplantation. Pancreatic islets were camouflaged with multi-branched PEG (6-arm-PEG-catechol) for minimizing of host immune reaction. It effectively inhibited xenogeneic immune reaction, because 6-arm-PEG-catechol could be more highly packed on the islet surface compared to linear PEG. Six-arm-PEG-catechol was covered the whole area of islet surface and surface camouflage technology did not affect the viability and functionality of islets. In addition, the synergistic effects of surface camouflage on immunoprotection for transplanted islets with low doses of immunosuppressive drugs, such as tacrolimus and anti-CD154 mAb, were established in the xenotransplantation model. When the recipients of 6-arm-PEG-catechol grafted islets were injected with 0.2 mg/kg of tacrolimus and 0.1 mg/mouse of anti-CD154 mAb, normal glucose level was maintained up to 50 days of transplantation without any fluctuation of glucose level. Therefore, a newly developed protocol using 6-arm-PEG-catechol with tacrolimus and anti-CD154 mAb would certainly be an effective combination therapy for the treatment of type 1 diabetes.

To investigate the inhibition effects of pancreatic islet transplantation on the progression of obese type 2 diabetes, we analyzed the effects of surface camouflaged islet transplantation on delaying the disease progression in a *db/db* diabetic mouse model. Surface camouflaged islets using 6-arm-PEG-

catechol were transplanted in *db/db* type 2 diabetic mice. The fat accumulation and toxicity in the liver, the expansion of islets in the pancreas, and the size change of abdominal adipocyte were analyzed. In addition, the blood glucose control, insulin levels and immunohistochemical staining of recovered tissues were analyzed after transplantation. Then co-administration of anti-CD154 mAb and tacrolimus (IT group) deterred the pathophysiological progression of obese type 2 diabetes. At day 3 of transplantation, the serum insulin concentration of IT group was increased compared to the control group, with the euglycemic control. The immunohistochemical studies demonstrated that the mass of 6-arm-PEG-catechol grafted islet was preserved in the transplantation site for 14 days; however, the intensity of insulin staining and serum insulin level was decreased with time. Therefore, transplantation of 6-arm-PEG-catechol grafted islets in the kidney capsule of *db/db* diabetic mice prevented the progression of obese type 2 diabetes and reduced the blood glucose level when immunosuppressive drugs were co-administered.

Transplanted islets were eventually rejected by host immune reaction with time. Thus, repetitive islet transplantation is needed to treat type 1 diabetic patients experiencing graft rejection. The secondly transplanted islets might be rapidly rejected due to sensitized immune reaction of the humoral and cellular immunities induced by the first transplanted islets. Thus, we explored whether the incorporation of PEG on the surface of the islets can be an affordable immunoprotective remedy for repeated islet transplantation. Unmodified islets transplanted in combination with cyclosporin A (CsA) and

anti-CD4 mAb (OX-38) into the sensitized recipients did not maintain a normal glucose level over 20 days. However, three of the five recipients became normoglycemic up to 30 days when PEGylated islets were transplanted in combination with CsA and anti-CD4 mAb. These results demonstrated that PEGylation alone was not an affordable immunoprotective method, but the combination of CsA and anti-CD4 mAb along with PEGylation showed a highly improved a synergic effects on the inhibition of sensitized host immune reactions.

In conclusion, surface camouflage and genetically engineered pancreatic islets were effective for treating diabetes mellitus. In the future study, we are expecting to achieve prolonged normoglycemia in diabetes patients using porcine islets in clinical studies.

Keywords: diabetes, islet transplantation, surface camouflage, genetic engineering, poly(ethylene) glycol, exendin-4

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Abbreviations

ALS	Anti-lymphocyte serum
AMPK	Adenosine 5'-monophosphate activated protein kinas
APCs	Antigen presenting cells
CsA	Cyclosporin A
CTLA-4	Cytotoxic T-lymphocyte antigen-4
Diabetes	Diabetes mellitus
DPF	Designated pathogen-free
DPP-4	Dipeptidyl peptidase-4
ES	Embryonic stem cells
Ex-4	Exendin-4
FITC	Fluorescein isothiocyanate
FK506	Tacrolimus
GLP-1	Glucagon-like peptide-1
GLUT-4	Glucose transporter type 4
IBMIR	Instant blood mediated inflammatory reaction
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase

IPGTT	Intraperitoneal glucose tolerance test
LV-SP-Ex-4	Lentiviral vector encoding for secretion signal peptide-linked Ex-4
mAb	Monoclonal antibody
MR-1	Anti-CD154 monoclonal antibody
NBG	Non-fasting blood glucose level
PEG	Poly(ethylene) glycol
PEG-lipid	Poly(ethylene glycol)-phospholipid
PPAR-γ	Peroxisome proliferator-activated receptor gamma
siRNA	Short interfering RNAs
SP-Ex-4	Secretion signal peptide linked Ex-4
SUR1	Sulfonylurea receptor 1
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor

Chapter 1. Introduction

1.1. Diabetes mellitus

Diabetes mellitus is one of the metabolic diseases in which a person has high blood glucose level due to insufficient production of insulin from islets or inadequate cell response to insulin secreted from islets (Fig. 1.1). The classical symptoms of diabetes are polyuria, polydipsia and polyphagia. There are three types of diabetes mellitus (diabetes). Type 1 diabetes patients do not produce the insulin from islets because they are destructed by their autoimmune reaction. This is referred to as "insulin-dependent diabetes mellitus" or "juvenile diabetes". Type 2 diabetes patients have insulin resistance; which cells cannot properly uptake the glucose from blood. This is referred to as "insulin-independent diabetes" or "adult-onset diabetes". Last one is called gestational diabetes is occurred when a women without previous diagnosed diabetes exhibit high glucose levels during pregnancy (Fig. 1.2). Type 2 diabetes is the most common type in the world, affecting 90% of U.S. diabetes population. After the discovery of insulin in late 1921, all forms of diabetes have been treatable. Since that time, different classes of diabetes drugs have been developed for the treatment of diabetes. Especially, type 2 diabetes patients were treated with oral hypoglycemic agents as primary treatment of disease. Although many treatment options are available for diabetes treatment, there are several aspects to take into consideration to improve patient's quality of life.

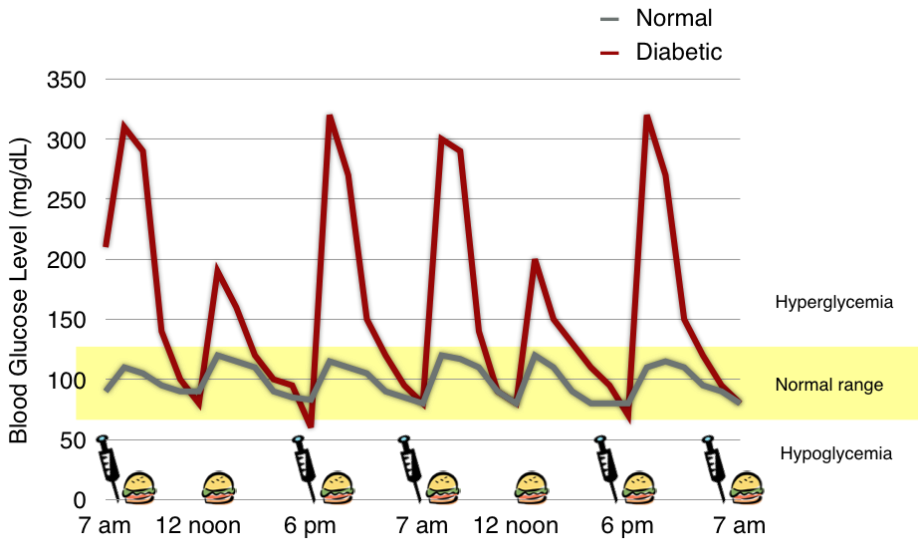
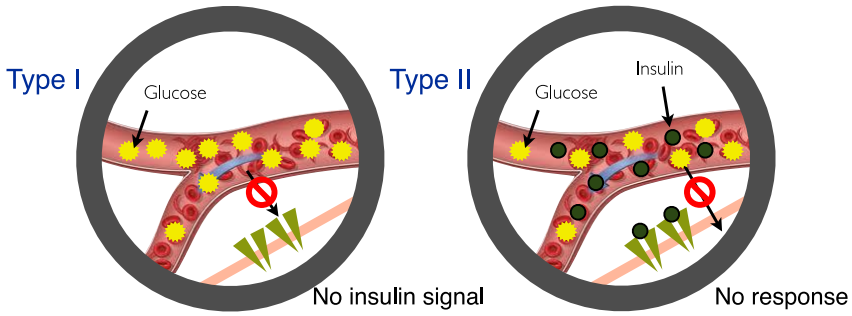


Figure 1.1. Schematic blood glucose level in diabetes mellitus patients



Features	Type 1 diabetes	Type 2 diabetes
Onset	Sudden	Gradual
Age at onset	Any age (mostly young)	Mostly in adults
Body habitus	Thin or normal	Often obese
Ketoacidosis	Common	Rare
Autoantibodies	Usually present	Absent
Endogenous insulin	Low or absent	Normal decreased or incresed
Concordance in identical twins	50%	90%
Prevalence	Less prevalent	More prevalent (90 to 95%)

Figure 1.2. Different features of type 1 and type 2 diabetes mellitus

(Modified Oct. 10 (2012) from website http://mixturesrx.com/blog/?tag=type-3-diabetes)

1.2. Symptoms and signs of type 1 diabetes mellitus

The typical circumstance of type 1 diabetes diseased patients is during the sudden weight loss (1 or 2 kg/week), accompanied by polyuria at night, and intensive thirst (polydipsia) in a young lean subject, 10-12 years of age. Overwhelming fatigue is always presented and patients' appetite is good. A general symptom is usually not very revealed, but these kinds of signs have been continued for more than several weeks, muscular atrophy is occurred. Especially, breath smell of acetone may be recognized in the presence of ketosis.

1.3. Treatment of type 1 diabetes mellitus

1.3.1. Insulin therapy

Insulin therapy is the most important part of the treatment of type 1 diabetic patients. Insulin was administered subcutaneously route using syringes, needles, and pen types (Fig. 1.3). Sometimes, external insulin infusion pumps are used in limited type 1 diabetic patients.

There are many different types depending on the onset of action. Rapid-acting insulin is injected before meals to stimulate prandial insulin secretion; intermediate or long-acting insulin is injected once or twice per day for regulating basal insulin level.

However, locally injected insulin therapy is limited due to erratic control, which induces peripheral hyperinsulinemia. In addition, systemic hypoglycemic or insulin shock can occur as a result of insulin therapy.

Furthermore, insulin therapy did not effectively inhibit the complications related to diabetes due to self-regulation of blood glucose level.

Needle and Syringe

- Most common, least expensive
- Covered by most insurance plans
- Allows mixing 2 or more kinds of insulin

Insulin Pen

- This device includes a needle and a cartridge of insulin
- Easy to measure the insulin and prepare the shot
- May attract less attention in public places

Insulin Infusion Pump

- Can deliver insulin very tiny amounts
- Expensive

Figure 1.3. Different types of insulin delivery devices

1.3.2. Complications of insulin therapy

Hypoglycemia is the most common complication of insulin therapy. It is commonly known that intensive insulin therapy increases the risk of hypoglycaemia in diabetic patients. Episode of hypoglycaemia was three times as frequents as in the conventional treatment group [1]. In addition, patients with uncontrolled diabetes experienced weight gain after starting insulin therapy, which is inevitable consequence of the anabolic action of hormones. It is due to restoration of fat and muscle bulk. Insulin oedema is not normally occurred at the start of insulin therapy in patients with poorly controlled diabetes, including those with ketoacidosis. The acute sodium and water retention cause oedema after proceeding long-term osmotic diuresis. Ephedrine has been suggested to be effective for refractory insulin oedema [2]. Local injection of insulin also causes lipohypertrophy. Lipohypertrophy is defined as local overgrowth of subcutaneous adipose tissue in response to the lipogenic and high local insulin concentration. It is generally occurred in patients with multiple daily insulin injection in the same site. Injecting in the same place can cause hard lumps or extra fat deposits to develop. lipohypertrophic effect can cause poor blood glucose control because insulin absorption is impaired in subcutaneous area. Furthermore, some impure component and insulin from animal pancreases is often immunogenic and these immunogenic components (pancreatic proteins, polypeptide, or retardant agents, or the insulin itself) induced the immune reaction in some patients.

1.4. Islet transplantation

1.4.1. History of islet transplantation

The first person attempted to graft pancreatic tissue to cure diabetes was named Charles Pybus (1882-1975). However, the recent era of islet transplantation research was credited to Paul Lacy's studies dating back more than three decades, Lacy's work described a novel collagenase-based method to isolate islets and opened the way for a new era of intervention by islet transplantation therapy [3]. Since that time, many studies demonstrated the potential application of islet transplantation to cure diabetes in both rodents and non-human primates [4, 5]. In addition, since the time Lacy commented on the possibility of "islet cell transplantation as a therapeutic approach for the possible prevention of the complications of diabetes in man" [6] during the Workshop on Pancreatic Islet Cell Transplantation in Diabetes, there has been tremendous progression in islet isolation techniques and immunosuppressive regimens resulted in the first human islet transplantation clinical trials. The first successful trial of human islet allotransplantation resulting in long-term reversal of diabetes was performed at the University of Pittsburgh in 1990 [7]. However, despite scattered signs of improvement, restoration of normal blood glucose level in diabetic patients in the late 1990s was hardly achieved. In 2000, Dr. James Shapiro and colleagues showed that glucose control could be achieved with islet transplantation and steroid-free immunosuppressive drugs [8] Then, they published the first paper on the "Edmonton Protocol" in the New England Journal of Medicine. This

Edmonton protocol has been used by islet several transplant centers and has considerably increased islet transplant success. Recently, B. Hering et al reported high insulin independence rate with single-donor islet infusions after slight modification in the experimental procedures [9, 10]. Currently, there are many successful islet transplantation cases for curing of type 1 diabetes.

1.4.2. Research scope of islet transplantation

1.4.2.1. Instant blood-mediated inflammatory reaction

After transplantation into portal vein, a considerable amount of islets is lost in the immediate post-transplant period due to non-specific inflammation (instant blood-mediated inflammatory reaction (IBMIR)) [11]. IBMIR is triggered by coagulation, complement activation, platelet aggregation and monocytes infiltration. In addition, islet surface molecules such as tissue factor, collagen residues and inflammatory factors induce the IBMIR after transplantation. Clinical allotransplantation results revealed that 70% of the transplanted islets are lost during the early stage of transplantation [12] (Fig. 1.4).

To prevent early graft loss, there are three types of interventional strategies. First strategy consists of the systemic administration of drug to the recipient such as heparin, low-molecular-weight dextran sulfate [13-17], nacystelyn [18-20], melagatran [17, 21], anti-TNF-alpha monoclonal antibody [22], 15-deoxyspergualin [23, 24], activated protein-C [25, 26], and statins [27, 28] to treatment about IBMIR. Another strategy is pre-treatment of

anti-oxidant or coagulation drugs to isolated islets prior to transplantation [29, 30]. The other method is genetic modification of islets to reduce of inflammation and coagulation [31, 32].

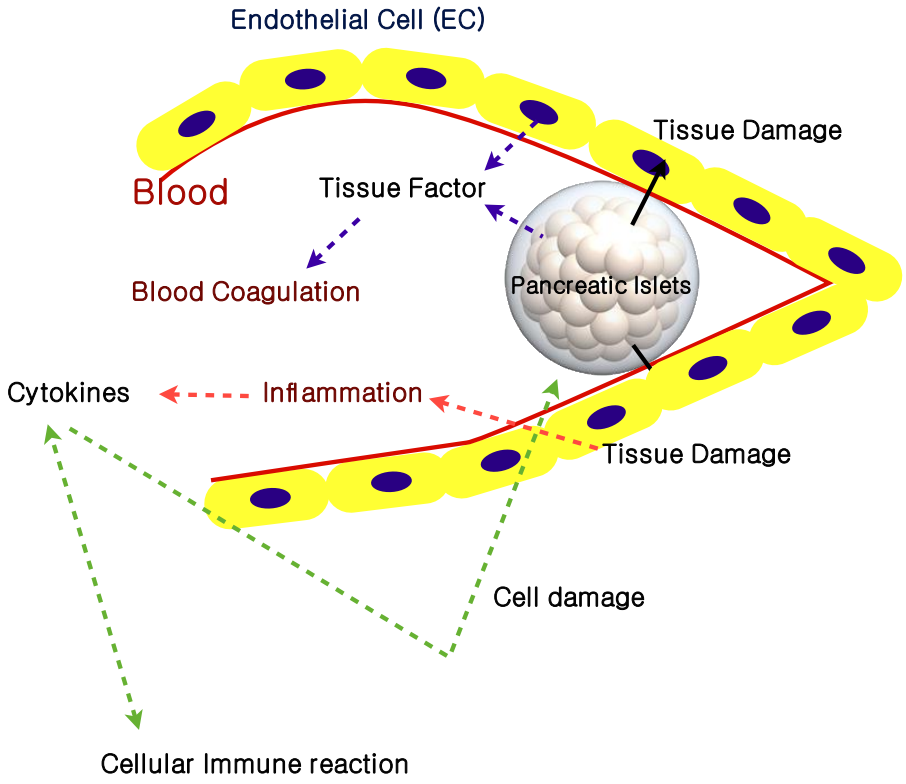


Figure 1.4. Instant blood-mediated inflammatory reaction (IBMIR) after intraportal delivery of islets

1.4.2.2. Immune reaction in islet transplantation

Firstly, transplanted islets are damaged by non-specific inflammation after transplantation. Then, antigen-presenting cells (APCs) undergo maturation and become activated by transplanted islets since these are recognized as antigens. In addition, APCs migrate to draining lymph nodes and stimulate the activation of T cells, resulting in increasing numbers of anti-donor T cells. Finally, migrated T cells will induce cytotoxicity to grafted islets [33] (Fig. 1.5).

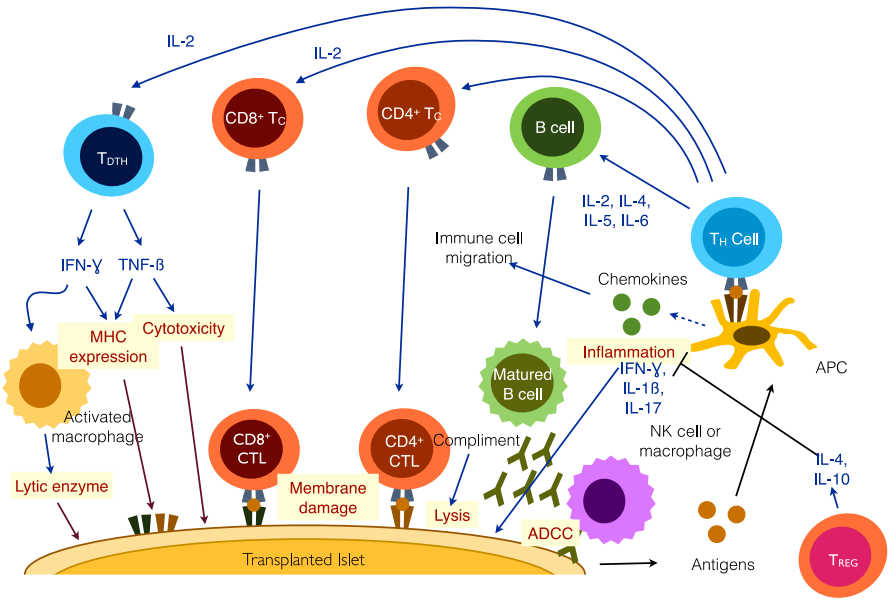


Figure 1.5. Illustration showing islet immune response after islet transplantation

(Modified from T.J. Kindt, R.A. Goldsby, B.A. Osborne, and J. Kuby, *Kuby immunology* (Ch.17, Fig. 17-6), W.H. Freeman, New York, 2007.)

1.4.2.3. Immunosuppressive therapy

Suppressing the immune response with cocktailed immunosuppressive drugs can prevent transplant rejection. Azathioprine, glucocorticoids, and anti-lymphocyte serum (ALS) were included in the first generation. Azathioprine is a calcineurin inhibitor. Cacineurin is a calcium-dependent serine/threonine phosphatase protein in the cytosol that removes phosphates from cytoplasmic regulatory protein. Then, regulatory protein penetrates the nucleus and performs as transcription factors. Thus, inhibition of calcineurin activity occurs to alleviation of production of cytokines. Especially, IL-2, essential cytokines for activation of T cells, production is inhibited. However, these kinds of drug have significant side effects such as nephrotoxicity, hypertension, hepatotoxicity, neurotoxicity, hirsutism, gingival hyperplasia, and gastrointestinal toxicity [34]. Glucocorticoids are also widely used agents for immunosuppression. These drugs effective inhibits the proliferation of T cells and expression of genes encoding for specific cytokines. Furthermore, they block the production of IL-2 and effectively inhibit the non-specific inflammatory reaction. Unfortunately, these drugs also have many side effects such as ulcers, hyperglycemia, osteoporosis, and infection when the recipients were administrated with these drugs for a long time [35, 36]. Anti-lymphocyte serum is also widely used for inhibition of immune reaction during organ and islet transplantation [37].

Second generation drugs are still used for immunosuppression, which are cyclosporine and tacrolimus (FK506) [38]. Cyclosporine makes the complex with cyclophilin, cytoplasmic receptor protein, which forms the

heterodimeric complex. These complex acts on T cells. FK506 binds to protein called FK506-binding protein in the cytosol [39]. However, these second generation drugs also have toxic effect to islets and diabetogenic effects. Newly developed agents, e.g., FTY720 [40] and lisofylline [41] improve the survival time of grafted islets with novel combinations of existing agents.

At University of Alberta in Edmonton, they reported a successful immunosuppressive drug therapy protocol in allotransplantation. The patients were sequentially transplanted with islets 2 to 10 weeks apart using two or more pancreases to achieve adequate mass of islets and administrated with a glucocorticoid-free immunosuppressant regimen which are sirolimus (rapamycine), IL-2 receptor antibody (daclizumab), and tacrolimus [8]. They reported that the rate of insulin independence for 1 year of transplantation was about 80%. However, after 5 years of transplantation, only below 10% of patients under the Edmonton protocol achieved insulin independence [42].

Recently, immune modulation and tolerance inducing drugs were used for inhibition of islet graft loss. Representative method of tolerance inducing is co-stimulation blockade drugs such as cytotoxic T-lymphocyte antigen-4 (CTLA-4) immunoglobulin (Ig) [43] and anti-CD154 monoclonal antibody (anti-CD154 mAb) [44]. CTLA-4 is a potent T cell down regulatory molecular which expresses for the development of peripheral tolerance. Thus, CTLA-4 Ig can improve the allograft survival by interfering between B7-1 and B7-2, and CD28 or CTLA-4-mediated co-stimulation by competitive binding on the APCs. Crucial procedures of T cell activation involves the

binding between expressed CD40 on the APCs and CD154 or CD40 ligand on the T cells. Thus, anti-CD154 mAb effectively inhibits T cell activation and consequently improves the increasing of graft survival rate [44-46] (Fig. 1.6, Table 1.1).

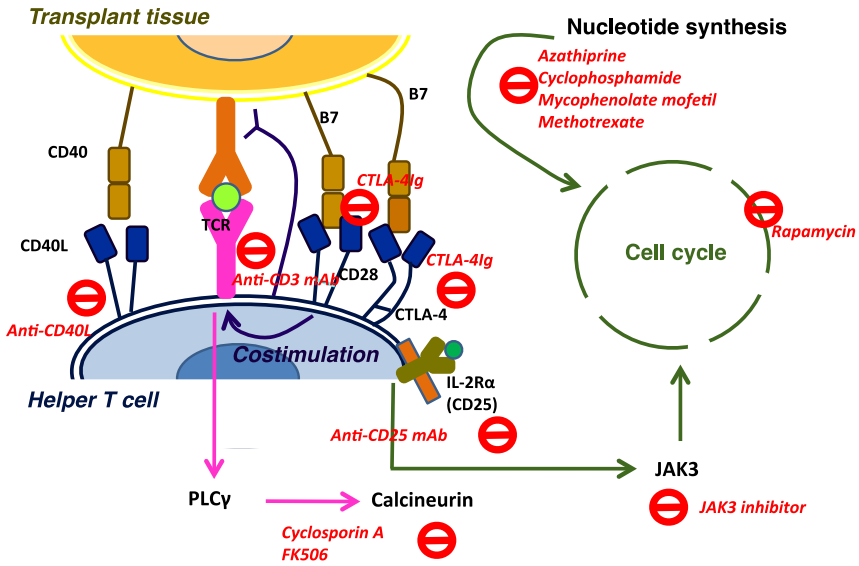


Figure 1.6. The mechanism of action; Immunosuppressive drugs

(Modified from T.J. Kindt, R.A. Goldsby, B.A. Osborne, and J. Kuby, *Kuby immunology* (Ch.17, Fig. 17-10), W.H. Freeman, New York, 2007.)

Table. 1.1. List of Immunosuppressive drugs

Immunomodulators: Immunosuppressive drugs				
Intracelluar (initiation)	Antimetabolites			Purine synthesis inhibitor: Azathioprin, **Mycophenolic acid** Pyrimidine synthesis inhibitors: **Leflunomide**, Teriflunomide Antifolate: Methotrexate
	Macrolides/other IL-2 inhibitors			FKBP/Cyclophilin/Calcineurin: **Tacrolimus**, **Cyclosporin**, Pimercrolimus
	TNF inhibitor			Abetimus, Lenalidomide
Intracellular (reception)	IL-1 receptor antagonists			Anakinra
	mTOR			**Sirolimus**, Everolimus, Ridaforolimus, Temsirolimus, Umirolimus, Zotarolimus
Extracellular	**Antibodies**	Monoclonal	Serum target (noncellular)	Compliment component 5 (Eculizumab), TNFs (Infiximab, Adalimumab, Certolizumab pegol, Afelimomab, Golimumab), Interleukin 5 (Mepolizumab), Immunoglobulin E (Omalizumab), BAYX (Nerelimomab), Interferon (Faralimomab), IL-6 (Elsilimomab) IL-12 and IL-23 (Lebrikizumab, Ustekinumab)
			Cellular target	**CD3** (Muromonab-CD3, Otelixizumab, Visilizumab), CD4 (Clenoliximab, Keliximab, Zanolimumab), CD11a (Efalizumab), CD18 (Erlizumab), CD20 (Afutuzumab, Rituximab, Ocrelizumab, Pascolizumab), CD23 (Gomiliximab, Lumiliximab), CD40 (Teneliximab, Toralizumab), CD62L/L-selectin (Aselizumab), CD80 (Galiximab), CD147/Basigin (Gavilimomab), **CD154** (Ruplizumab) Blys (Belimumab), **CTLA-4** (Lipilimumab, Tremelimumab), CAT (Bertilimumab, Lerdelimumab, Metelimumab), Intergrin (Natalizumab), Interleukin-6 receptor (Tacilizumab), LFA-1 (Odulimomoab), **IL-2 receptor/CD25** (Basiliximab, **Daclizumab**, Inolimomab), T-lymphocyte (Zolimomab aritox)
			Unsorted	Atorolimumab, Cadelizumab, Fontolizumab, Maslimomab, Morolimumab, Pexelizumab, Resizumab, Rovelizumab, Sipizumab, Talizumab, Telimomab aritox, Vapaliximab, Vepalimomab
		Polyclonoal	Anti-thymocyte globulin, Anti-lymphocyte globulin	
	-cept (Fusion)	CTLA-4 (Abatacept, Belatacept), TNF inhibitor (Etanercept, Pegsunercept), Aflibercept, Alefacept, rilonacept)		

(Modified Oct. 10 (2012) from website http://en.wikipedia.org/wiki/Immunosuppressive_drugs)

1.4.2.4. Inadequate islet supply and its solution

An inadequate supply of islets represents a critical barrier to islet transplantation. One of the promising solutions to this problem is the use of cells derived from embryonic (ES) or adult stem cell. Possibility of growing a limitless number of cells that already have the ability to become functioning endocrine tissue makes this approach attractive. In addition, there is possibility to do autologous therapies using adult stem cells, thereby circumventing host immune reaction. A possible alternate source of islets is through the controlled differentiation of stem or precursor cells. ES cells offer several advantages including multi-potency and the possibility of being non-immunogenic. Some improvement has been made in the derivation of insulin-producing cells from ES cells, however problem in aiming homogenous differentiation must be overcome before this approach can be clinically applicable. Another approach is the isolation and exploitation of islet progenitor cells from the adult pancreas. Evidence proposes that the pancreatic ductal-epithelium may contain islet precursors. But, up to the present time, no precise cellular phenotype has been known to have endocrine differentiation capability.

Recently, transplantation of porcine islets has been proposed as an alternative solution to overcome problems associated with inadequate islet supply. The quality of islets products from young, living, health, and designated pathogen-free (DPF) donor pigs would be predictably high and not compromised, as with human islet products, by co-morbidity, brain death, age and cold ischemia. In addition, genetically engineered pigs would present

opportunities for maximizing recipient immunosuppression. Thus, exploiting the unique possibilities related to porcine islet products would increase the availability and benefit-risk ratio of islet replacement therapies when compared to human islet sources.

1.5. Encapsulation and immunoisolation technologies

1.5.1. Macroencapsulation and microencapsulation

Immunoisolation of transplanted islets is a promising method that prevents the destruction of transplanted islet against to host immune reaction. Immunoisolation devices effectively inhibit the immune cell infiltration into transplanted islets. Immunoisolation devices generally used the semipermeable membrane for encapsulation of islets, which allows transportation of insulin, glucose, and nutrients but resists the immune cells and antibodies of related immune reactions. Physical barrier could effectively prevent the destruction of islets occurred by cellular and humoral-mediated immune reaction.

There are three types of encapsulation system for isolation of transplanted islets. First one is perfusion chambers directly connected to the blood circulation system (intravascular macrocapsules) [47].Another one is diffusion chamber in the tube or disk shapes that can be implanted to peritoneal cavity or subcutaneous site (extravascular macrocapsules) [48]. Third type is the encapsulation of several islets in globular membranes (extravascular microcapsules) [49]. Furthermore, recent hybrid systems using

both macrocapsule- and microcapsule-based technology is being investigated for reduce the problems associated to single system use.

Although the immunoisolation device can effectively inhibit the destruction of islets against to host immune system, there are many impediments furthering clinical research. In case of intravascular macrocapsule system, this device requires intensive systemic anticoagulation due to the direct contact to foreign materials with blood and the possibility of blood coagulation, inducing potentially fatal blood clot formation. In another word, device is low biocompatible material when implanted into patients. In addition, surface fibrosis and biocompatibility are also part of the most significant barriers perceived to prevent the successful use of extravascular macrocapsule system. Alginate encapsulation is most common method of extravascular microcapsule system. Even though alginate is the most common biomaterial and does not induce the surface fibrosis when compared with other isolation devices, it is limited due to the purity of alginate [50]. Also cell viability and functionality might be affected because of the size limits mass transport across the capsule membrane (Fig. 1.7). Currently, Living Cell Technology (New Zealand, Manukau) is pursuing clinical studies on alginate microcapsule for pancreatic porcine islet cell graft immunoprotection for the treatment of type 1 diabetes.

Other types of devices for islet transplantation have been developed. Amicon XM50 is a hollow fiber made from poly(acrylonitrile-co-vinyl chloride) and it has been used for encapsulation of neonatal mouse islet cells before transplantation [51]. Islet Sheet Medical (San Francisco, CA) is an islet

sheet containing islet, mesh and alginate. A central core region contains islets suspended in alginate; this and a reinforced mesh are surrounded bonded to acellular immune-protective alginate layers [52]. Recently, they tried to apply islet sheet medicals to large-animal.

All of these kinds of devices have chronic problems related to cell viability. In addition, only abdominal cavity or subcutaneous areas are suggested as an optimal site for transplantation (Fig. 1.8). Thus, apparent clinical studies are limited for future application.

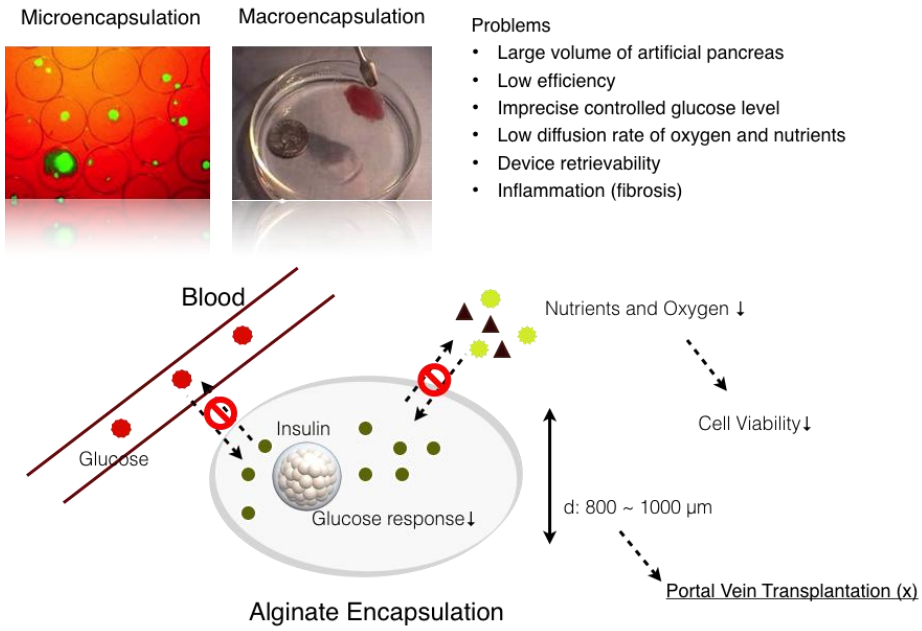
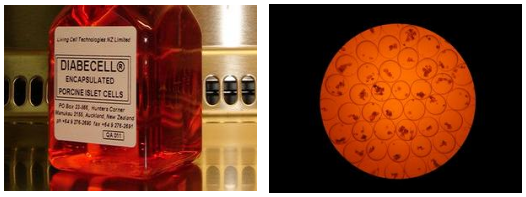
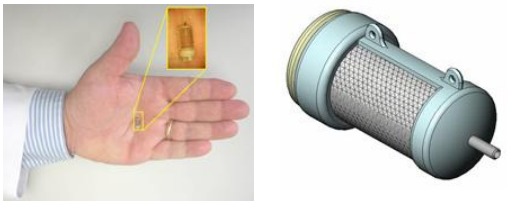


Figure 1.7. Limitations of microencapsulated and macroencapsulated islets

Islet Sheet Medical (Islet Sheet)



Living Cell Technologies (Encapsulated Porcine Islets)



Bio Hybrid Technoloby Inc (Artificial Chamber)

Figure 1.8. Islet transplantation device for immunoprotection

(Retrived Oct. 31 (2012) from http://hanumanmedicalfoundation.org/type-1-diabetes-research/islet-sheet-project.html, http://www.lctglobal.com/Products-and-Services/ and Lanza RP, Sullivan SJ, Chick WL. Perspectives in diabetes. Islet transplantation with immunoisolation. Diabetes. 1992;41:1503-10.)

1.5.2. Advantage of surface modification

Surface camouflage of islet significantly differed from other encapsulation methods such as macro- or microencapsulation that cannot be applied to islet transplantation via portal vein due to their device sizes. The viability of encapsulated islets was consistently decreased in a time-dependent manner since encapsulated islets were affected by the emergence of oxygen and nutrient deficiency. However, the surface camouflage method did not induce the size increase of islets, thus effective supply of oxygen and nutrients might be assured from outer environment to islet. In addition, no size increase provided accessible portal vein islet transplantation in clinical application. Furthermore, surface modification procedure is very simple and islets is incubated very short time with polymer in the buffer condition. Therefore, the viability of islets was not decreased during the modification (Fig. 1.9).

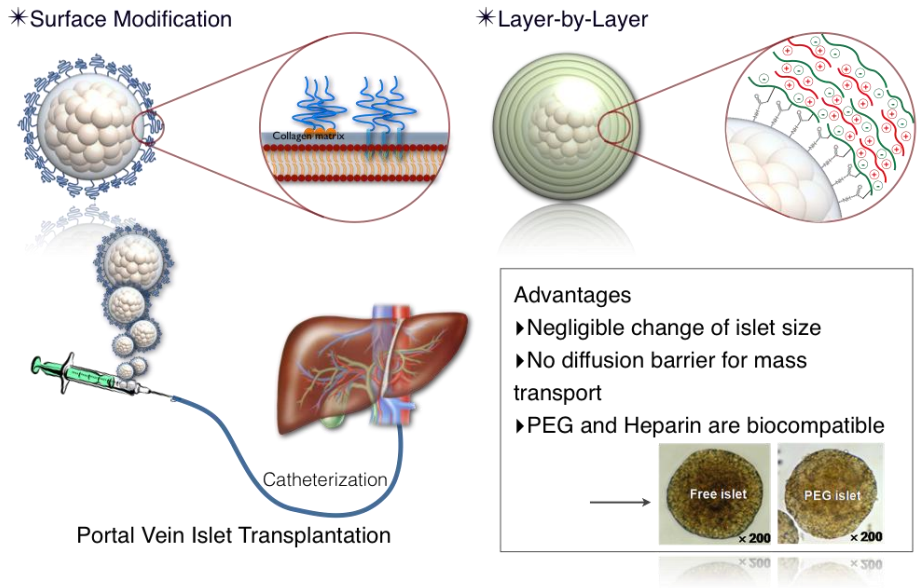


Figure 1.9. Advantages of mono-layer or layer-by-layer modified islets

1.5.3. History of surface camouflaged pancreatic islets

Surface camouflage of islets is an alternative approach to immunoisolation and immunoprevention. Surface modification process involves the hydrophilic chains such as PEG and heparin derivatives to the islet surface to obtain molecular encapsulation of the islets. The surface of islets has less resistance and reduced thickness diffusional barrier. A diffusional barrier over 200 μm in length is injurious to diffusive transport of nutrients and metabolites. Furthermore, surface camouflaging did not increase the total volume of tissue per IEQ, which makes transplanting islets into human subjects practicable through the portal vein.

Strategies for surface modification of islets initially use the linear types of hydrophilic PEG with an activated functional group. PEG has been used for surface modification of islets for inhibition the immune cell adhesion and infiltration. In the previous study, the condition of PEG grafting to islet surface was optimized [53]. In addition, a combination therapy of PEGylation and cyclosporin A was succeeded for islet allotransplantation to treat type 1 diabetes [54]. Other research groups, Panza et al. reported rat islet surface coating technology and subsequently reported cytoprotection effect of PEG for porcine islet xenotransplantation in diabetic SCID mice [55, 56]. In addition, Xie et al. further reported the albumin grafting to islet surface using a disuccinimidyl derivative [56]. Teramura et al. has been studied the surface modification of cells and islets with thin polymer using PEG-conjugated phospholipid (PEG-lipid) and polyvinyl alcohol (PVA) [57]. They analyzed

the surface coverage of hamster islets using PEG-lipid labeled with the fluorescent dye FITC. In addition, they analyzed the effect of surface modification on the graft survival post portal vein transplantation [58]. Some damaged islets observed after one day. However, PEG-lipid grafted islets were not damaged and remained morphology in the blood vessels one day after transplantation. Immunohistochemistry data supported the finding that graft survival time of islet is prolonged after transplantation.

Our research group has performed several in *vitro* analyses of immunoportective effects of PEGylated islets after culturing them with immune cells [53]. When unmodified islets were cultured with immune cells, they were damaged after completely losing the integrity of islet morphology at day 7. However, PEGylated islets were not destructed by co-culture with immune cells. Thus, cytokine level of IL-2 and TNF-alpha from immune cells were highly secreted when cultured with unmodified islets. In addition, the degree of immunological response of PEGylated islets after transplantation was quantitatively and qualitatively analyzed in the previous study [59, 60]. Lee *et al*. demonstrated that PEGylation alone was not enough to improve the survival time of allotransplanted islets although the PEG effectively inhibited the immune cell infiltration into transplanted site. We assumed that the graft rejection is due to cytokine attack triggered by immune cells. Based on these findings, Lee *et al*. evaluated the clinical potential of a new immunoprotective therapy based on PEGylation and immunosuppressive drugs in an effort to minimize the required dose. A low dose of cyclosproin A (CsA, 3 mg/kg), was intravenously administered after unmodified and PEGylated islets

transplantation. In the case of unmodified islet transplantation, transplanted islets were subjected to rejection within 2 weeks after CsA administration. On the other hand, PEGylated islets survived more than 1 year and stably maintained normal blood glucose level [61].

In the future, the porcine islets will be used to solve the problems of tissue supply for islet transplantation in type 1 diabetes [62]. However, porcine islets are very fragile and the cell membranes are directly exposed on the outer layer of porcine islets. Thus, more potent surface modification technology is needed to prevent the immune reaction and rejection after transplantation.

1.6. Gene therapy for improving the success of islet transplantation

1.6.1. Revascularization of transplanted islets

Capillary density of pancreatic islets is ~10 times higher then that of the exocrine tissue area in the pancreas [63, 64]. However, this specialized vasculature of isolated islets was disrupted during isolation procedures, thus intimidating the survival of islets in the core area [65]. Delivery of oxygen and nutrients to islets, and revascularization of transplanted islets were needed to increase the survival rate after transplantation. One of the important approaches to improve the angiogenesis in the transplanted sites was to induce VEGF gene expression. VEGF is reported to increase vascular permeability, which is very important for retaining the function of endocrine cells in the

organ. In addition, VEGF play an important role in the revascularization process after islet transplantation. Zhang *et al*. reported that hVEGF gene transduction using adenoviral vector to islets was effectively elevation of VEGF expression and improve the revascularization [66]. In addition, Kim et al. reported that hypoxia inducible VEGF gene delivery to rat islets using polyethylenimine was very useful for the development of anti-apoptotic for islet transplantation [67].

1.6.2. Gene transduction for apoptosis inhibition of transplanted islets

Cytokine- and hypoxia-induced apoptosis is also a very important factor to affect the viability of transplanted islets. One of them, non-specific inflammatory reaction is occurred in the initial stage of transplantation. Investigators have identified the roles of macrophage in the biology of inflammation. Pro-inflammatory cytokiens such as TNF-alpha, IFN-gamma and IL-1 beta lead to the activation of inducible nitric oxide synthase (iNOS) and release the nitric oxide. Thus, IL-1 beta-receptor antagonist (IL-1Ra) transduction to islets prevented the cytokine-induced apoptosis [68].

Bcl-2 family also induce or inhibit the cytochrome C release into cytosol, which activates caspase-3 and caspase-9. Anti-apoptotic proteins such as Bcl-2 and Bcl-X prevent the apoptosis of transplanted islets. However, pro-apoptotic proteins such as Bax, Bak, Bad, and Bok promote apoptosis of transplanted islets. *Ex vivo* gene transfer of Bcl-2 has potential to minimize

the apoptosis of transplanted islets and reduce the islet mass for treatment of type 1 diabetes [69].

1.6.3. Gene silencing for islet transplantation

Gene silencing using short interfering RNAs (siRNAs) was suggested to improve the survival time of grafted islets. Silencing pro-apoptotic gene for islet viability or functionality is a promising method for better therapeutic efficacy in islet transplantation. siRNA against iNOS gene delivery to islets significantly prevent the apoptosis [70]. In addition, gene silencing of a caspase-3 involved in apoptosis pathway using siRNA has beneficial effect in inhibiting apoptosis in islet transplantation [71].

1.7. Research rationale

Islet transplantation offers the potential to improve glycemic control in type 1 diabetes mellitus patients. Replacement therapy of insulin is used to control of the blood glucose; however, hypoglycemic episodes are unavoidable. Even though glucose monitoring after exogenous insulin injection is performed daily, the unintended control of blood glucose levels in patients can be occurred, thereby cause many complications such as neuropathy, nephropathy, heart disease and retinopathy [72, 73]. Therefore, pancreatic islet transplantation is a promising strategy to treat type 1 diabetes, as it can achieve strict regulation of blood glucose level. However, antigens easily recognize the transplanted islets and they are susceptibly rejected by the host immune system. Activated macrophages stimulated the activation of T cells, and increased the secretion and expression of cytotoxic cytokines. Therefore, islet transplanted patients are needed to be injected with immunosuppressive agents such as daclizumab, tacrolimus, sirolimus, or CsA to prevent grafted islets from rejection. In 2000, Shapiro et al. reported successful results of islet transplantation using Edmonton protocol [8]. From 1 to 2 years after transplantation, 80-90% patients reached insulin independence. However, only 10% of recipients maintained the insulin independence after 5 years of transplantation [74, 75]. Recently, different techniques were used to improve the function and survival time of grafted islets. Recently, Hering et al. demonstrated high insulin independence rate with single-donor islet infusions after modification of pancreas preservation,

the culture medium, and peri-transplantation management, as well as, maintenance immunotherapies procedures [9, 10]. These protocols are currently limited by the need for lifelong immunosuppression. New strategy toward improving immunoprotection is in need to prevent transplanted islets since immunosuppressive drugs are known to cause many adverse effects.

Macroencapsulation and microencapsulation have been widely used for immunoprotection without immunosuppressive drug therapy. Although this strategy has been tested in animal models, several problems such as decreased cell viability and increased encapsulated islet size limited the clinical application. In addition, islet donor shortage is another hurdles revealed by the past islet transplantation studies. Several alternatives methods have been proposed to improve islet donor shortage, such as the porcine islets supply [44, 62, 76, 77] and stem cell therapy [78].

In this study, one method involves the exendin-4 (Ex-4) treatment, which is currently used to treat type 2 diabetes [79]. Ex-4 stimulates the glucose-dependent insulin secretion. Many studies have reported that Ex-4 protein treatment improved the blood glucose control in diabetic mice after transplantation [80]. Therefore, multiple Ex-4 injections into recipients have been proposed as a promising strategy to improve the function of transplanted islet. However, it should be noted, however, there are adverse effects such as emesis, nausea and pancreatitis [81, 82]. Thus we have designed a lentivial vector system capable of expression Ex-4 proteins to enhance islet cell function (Chapter 2).

To prevent the graft rejection against to host immune reaction,

surface camouflage of pancreatic islets using biocomparable PEG molecules have been proposed as an alternative approach of immune intervention instead of macro- and micro-encapsulation methods. Grafted PEG itself could not completely inhibit the host immune reaction, however, it was somewhat effective in inhibiting the infiltration and activation of immune cells. In the previous study, we have demonstrated that the graft survival time of surface camouflaged islet was improved in type 1 diabetes when the recipients were administrated with low dose of immunosuppressive drugs in allotransplantation model. The diabetic F344 rat model maintained normal glucose level over 1 year under the proposed protocol. Linear PEG derivatives were useful to inhibit the activation of allogeneic immune reaction. Recently, porcine islet transplantation to non-human primate model got spotlight about curing of type 1 diabetes The structure of insulin secreted from pig islets is similar to human insulin and they are different in one amino acid sequence and the source is available in reasonable quantities. In addition, islets isolated from porcine islets were well managed. Based on these facts, it is important to notice that immunoprotection of islets in xenogeneic hosts is a very important issue for successful islet transplantation. In this study, we have proposed powerful immunoprotective materials for surface modification of islets using multi-branched PEG derivatives (Chapter 3.)

Most obese type 2 diabetes show an insulin resistance in the early stage of the disease [83]. As the disease progresses, the functions of pancreatic beta cells are gradually deteriorated, finally developing severe insulin deficiency [84]. In these respects, we expected that additional exogenous

insulin administration might compensate for excessive demand for insulin secretion from the beta cells, and furthermore, deter the progression of liver damage in type 2 diabetes. Thus, basal-bolus insulin therapy is one of the methods for controlling the blood glucose level of type 2 diabetic patients, offering a way to closely stimulate natural insulin delivery in the clinic [85, 86]. However, the insulin therapy has shown many side effects with respect to hypoglycemia, patient willingness, and incompliance. Therefore, islet transplantation has been shown to be another alternative by which to properly deliver insulin to diabetic patients. Many researchers have reported that islet transplantation is effective for the treatment of mouse model of type 2 diabetes [87-93]. Unfortunately, it was hard to effectively deliver the insulin to type 2 diabetic patients due to the host immune reaction. Thus, we applied the combined immunoprotection protocol using polymer therapy (6-arm-PEG-catechol) and low dose of immunosuppressive drugs (Tacrolimus and anti-CD14 mAb) to treat *db/db* type 2 diabetic mice model (Chapter 4).

In spite of continuous administration of immunosuppressive drugs to prevent host immune reactions, islet graft failure may still ensue and transplantation must be repeated. However, secondly transplanted islets might be rapidly rejected due to sensitized immune reactions induced by the first transplanted islets. Thus, we have evaluated whether a surface camouflaging strategy would be effective in recipients that had been sensitized by a repeated islet allotransplantation (Chapter 5) (Fig. 1.10).

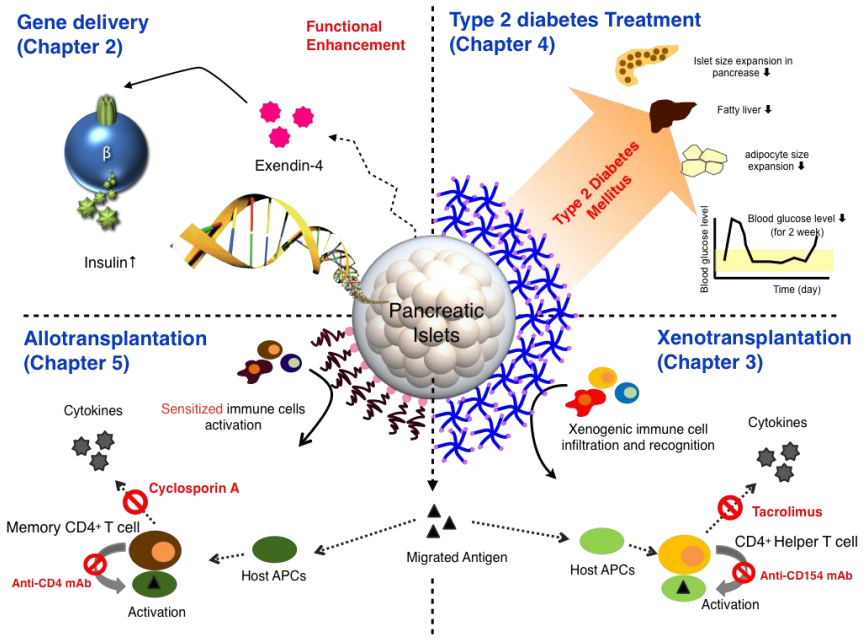


Figure 1.10. Illustration of islet transplantation for the treatment of diabetes mellitus using surface modification and genetic engineering

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Chapter 2. Gene Transduction of Secretion Signal Peptide-linked Exendin-4 to Pancreatic Islet for Enhancing Beta Cell Function

2.1. Introduction

Transplanting isolated islets from donor pancreas has been intensively studied as a possible long-term cure for diabetes [1, 2]. Previous studies have shown some promise in the field of islet transplantation; research must now be directed toward overcoming the hurdles revealed from the past transplant studies, such as the limited availability of cadaveric pancreas, shortage of islet donors, immune rejection and autoimmunity in diabetic patients [1, 3, 4].

Several alternatives have been proposed to improve islet donor shortage, such as the use of porcine islets for xenotransplantation [5-8] and stem cell therapy [9]. One method involves the use of exendin-4 (Ex-4) protein, which is currently employed in the treatment of type II diabetes [10]. Ex-4 evidences triggers a variety of gluco-regulatory actions via stimulating the glucose-dependent insulin secretion, the suppression of glucagon secretion and the stimulation of pancreatic beta cell proliferation and/or neogenesis [11, 12]. Additionally, Ex-4 has been shown to inhibit cytokine- [13-15] and hypoxia-induced apoptosis [16], and performs a role in preventing inflammation [17]. In this respect, many previous studies have demonstrated the efficacy of treating Ex-4 in islet transplantation to cure type I diabetes. A previous study has shown that, islet pre-cultured with Ex-4 had a higher types I diabetes curing rate than islets cultured without the drug in a syngeneic mouse model. [18]. In addition, Sharma et al. reported that Ex-4 treatment improved the metabolic control in streptozotocin (STZ)-induced diabetic mice after transplantation. [19]. Thus, Ex-4 treatment to islets or recipients was

effective in improving the graft survival time. However, ex *vivo* treatment of Ex-4 was not sufficient to maintain the improved islet function. Therefore injection of commercially available multi-therapeutic Ex-4 has been proposed as a potentially promising strategy to improve beta cell function. However, many side effects such as emesis, nausea, and weight loss have been associated to systemic and chronic administration of Ex-4 [20, 21].

In this study, to overcome current limitations, we have designed a genetically engineered lentiviral vector (LV) system for the secretion of the Ex-4 from the transplanted islets as an alternative method for the local delivery of the Ex-4. Ex-4 could be expressed from the inserted Ex-4 gene, but to ameliorate its secretion, the end of the Ex-4 gene construct was linked with a secretion signal peptide (SP) [10] to allow for the secretion of Ex-4 from the islets. We evaluated the effect of Ex-4 gene transduction on enhancing beta cell function and conducted further investigations to determine whether secretion signal peptide linked Ex-4 (SP-Ex-4) transduced islets could prevent cytokine- and hypoxia-induced apoptosis. Finally, it was evaluated in STZ-induced type I diabetic animal models whether the enhanced secretion of SP-Ex-4 could attenuate the essential number of islets required to treat diabetes *in vivo* as a long-term therapeutic option for diabetes.

2.2. Materials and Methods

2.2.1. Preparation of lentiviral vector encoding secretion signal peptide-linked Ex-4 (LV-SP-Ex-4)

The chemically synthesized Ex-4 cDNA was inserted into the pβ vector. The DNA fragment encoding for the SP from Ex-4 was also chemically synthesized and inserted into pβ-Ex-4 to create pβ-SP-Ex-4. The sequence of SP was as follows; 5' – ATGAAGATCATCCTGTGGCTGTGT GTGTTCGGCCTGTTCCTGGCCACCATGTTCCCCATCAGCTGGCAGA TGCCCGTGGAGTCGGCCTGTCCTCCGAGGACTCCGCCAGCTCCGAG AGCTTCGCC-3'. A furin cleavage site (RGRR) was inserted between SP and the Ex-4 cDNA. Since the SP of the expressed SP-Ex-4 protein was deleted in the Golgi apparatus, Ex-4 protein could be secreted as an original structure. The SP-Ex-4 fragment was amplified from pβ-SP-Ex-4 using a following primer set: the forward primer was CGGAATTCATGAAGATCA TCCTGTGGCTG and the reverse primer was GCGATATCTCACGATGG CGGAGG. Then the PCR gene fragment was inserted into the EcoRI site of the transfer vector, pHR'pgk-eGFP.

The recombinant lentivirus vector now contains three components (1:1:1 ratio): the transfer vector, pHR'CMV-SP-Ex-4-pgk-eGFP, containing an enhanced gene for the green fluorescent protein (eGFP) marker protein controlled by the pgk promoter, the packaging plasmid pCMV8.91 encoding Gag, Pol, Tat and Rev proteins for virus assembly, and the envelope plasmid pMD.G expressing the vesicular stomatitis virus glycoprotein (VSV-G) envelope protein. The virion particles, namely, LV-SP-Ex-4 or LV-Ex-4, were produced in 293T cells by transient Lipofectamine Plus (Invitrogen Co., Carlsbad, CA) co-transfection. The culture supernatant containing viral vector particles was harvested 48 h after transfection, clarified with a 0.45 μm

membrane filter (Nalgene Nunc, ThermoFisher Scientific, Rochester, NY), and immediately stored in a deep-freezer at -70℃. The virus titer was prepared by transducing HEK 293T and HeLa cells and the virus titer used in subsequent experiments was approximately 10^7 TU/ml. Isolated islets were infected with LV-SP-Ex-4 or LV-Ex-4 in DMEM for 15 h at 37^{o}C at a multiplicity of infection (MOI) of 12.5. Then, islets were washed with RPMI-1640 containing 1% of penicillin/streptomycin, and incubated in the same medium containing 10% of fetal bovine serum for 3 days before transplantation. The amount of Ex-4 secreted from the islets was measured using an enzyme immunoassay kit (Phoenix Pharmaceuticals Inc., Burlingame, CA).

2.2.2. Functionality and viability of SP-Ex-4 transduced islets

Pancreatic islets were isolated from outbred male Sprague Dawley (SD) rats by collagenase (Sigma, St. Louis, MO) digestion of the pancreas and discontinuous Ficoll™ PM400 (Amersham Biosciences AB, Uppsala, Sweden) density gradient centrifugation, and then finally hand-picked. Isolated islets were then cultured for 3 days in the RPMI-1640 (Sigma) culture medium, containing 10% fetal bovine serum (FBS; Sigma) at 37℃ under the humidified atmosphere containing 5% CO_2. Eight hundred of isolated islets were treated with 1 ml of LV-SP-Ex-4 or LV-Ex-4 for 15 h (MOI = 12.5). The concentration of applied lentivirus was 10^7 IU/ml.

The functionality of the transduced islets was evaluated by monitoring

the amount of insulin secreted from the islets in a Krebs-Ringer bicarbonate buffer (pH 7.4), containing low (2.8 mM) and high (28 mM) glucose concentration, respectively. The amount of secreted insulin was measured using a rat/mouse insulin ELISA kit (Millipore). The SI value was calculated by dividing the amount of insulin secreted at a high glucose solution by that secreted at a low glucose solution.

The viability of genetically modified islets was quantitatively analyzed using a cell counting kit, (CCK)-8 (Dojindo Molecular Technologies Inc., Rockville, MD). Briefly, 100 μl of the islet suspension (10 islets/well) was dispensed into a 96-well culture plate and treated with 10 μl of WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazoium, monosodium salt]. After 4 h of incubation, the absorbance was read at 450 nm using a microplate reader. To qualitatively analyze the viability islets, they were suspended in Hank's balanced salt solution (HBSS) containing 0.67 μM of acridine orange (AO, Sigma) and 75 μM of propidium iodide (PI, Sigma) for 4 min in dark condition. After washing with HBSS, the stained islets were observed under a fluorescence microscope (Nikon Eclipse TE2000S, Tokyo, Japan). Islets were defined as viable islets if more than 90% of cells showed green fluorescence.

2.2.3. Cytokine- or hypoxia-induced apoptosis in LV-SP-Ex-4 transduced islets

LV-SP-Ex-4 transduced islets and the control islets were cultured in

RPMI-1640 (Sigma) culture medium, containing 10% FBS at 37^{o}C under humidified atmosphere containing 5% CO_2 for 3 days. After washing, islets were again cultured with RPMI-1604 medium containing IL-1ß (100 U/ml; Pepprotech), TNF-α (1000 U/ml; Pepprotech) and IFN-γ (1000 U/ml; Pepprotech) for 3 days. Then the prevention effect of Ex-4 on cytokine-induced apoptosis was analyzed using a complementary technique of Annexin V staining and caspase 3/7 assays. FITC-Annexin V stained islets were dissociated using Trypsin-EDTA and the rate of apoptosis was determined using flow cytometry (BD FACS Calibur™, BD Biosciences, NJ). Incubated islets with cytokines were washed two times using HBSS, and the caspase 3/7 activity was measured using Caspase-Glo ® assay solution (Promega, Madison, WI). The intensity of luminescence was measured using microplate luminometer (Berthold Technologies, Germany). On the other hand, hypoxia-induced apoptosis was carried out as follows; LV-SP-Ex-4 transduced and untransduced islets (100 IEQ/well) were cultured under hypoxic (1% O_2 and 5% CO_2) or normoxic condition (20% O_2 and 5% CO_2) for 24 h, then the anti-apoptosis effect of Ex-4 was analyzed using CCK-8 and caspase 3/7 assays as mentioned above.

2.2.4. LV-SP-Ex-4 transduced islet transplantation in mice

Inbred male BALB/c nude mice and C57BL/6 mice, all of which were 7-8 weeks of age, were used as recipients. They were purchased from Japan SLC (Hamamatsu, Japan) and Orient Bio Inc. (Sungnam, South Korea),

respectively, and were housed under a specific pathogen-free condition. Diabetes mellitus was induced in the BALB/c nude mice and C57BL/6 mice by a single intraperitoneal (IP) injection of 220 mg/kg and 180 mg/kg of streptozotocin (STZ) (Sigma), respectively. Mice that had the glucose level over 350 mg/dl were selected as transplant recipients. All surgical and experimental procedures were performed according to the guidelines of the Institute of Laboratory Animal Resources, Seoul National University (IACUC no. SNU-070822-5).

STZ-induced diabetic recipients were anaesthetized by single IP injection of ketamine (80 mg/kg) and xylazine (16 mg/kg). The left kidney was exposed through a lumbar incision, and the left kidney capsule was incised and the islets were transplanted on the caudal outer surface of the left kidney. LV-SP-Ex-4 transduced islets or untransduced islets were transplanted into BALB/c nude mice and C57BL/6 mice. The non-fasting blood glucose concentration and the body weight of the BALB/c nude mice implanted with different amounts of islets were monitored every 2 or 3 days by drawing blood from the tail veins using a portable glucometer (Super glucocard II, Arkray, Kyoto, Japan). If the blood glucose concentration was higher than 200 mg/dl for two consecutive days, the islets were considered rejected. Intraperitoneal glucose tolerance test (IPGTT) using 20% glucose solution (10 μl/g) was performed to evaluate the glucose responsiveness of the transplanted islets at day 30 of transplantation. At the end of this experiment, nephrectomy was carried out for histological analysis.

Seven days after islets transplantation into C57/BL6 mice,

transplanted islets were harvested and the mean cross-sectional area of individual beta-cell (sectioned into 10-μm sections) was calculated. A measure of beta-cell area was determined by insulin antibody staining. The insulin positive cell area was calculated by ACT-2U imaging software (Nikon, Tokyo, Japan). In addition, serum insulin level in recipients was measured at day 14 of transplantation and was calculated using rat/mouse insulin ELISA kit (Millipore).

2.2.5. Immune response test of virus transduction

To evaluate the immune response caused by viral vectors, splenocytes were isolated from the spleen of the C57BL/6 mice at day 5 of transplantation. Splenocytes were isolated from the spleen. Briefly, the isolated spleen was placed into a cell strainer. The spleen was mashed through the cell strainer into the culture dish using the plunger end of the syringe. Cells collected from the culture dish were suspended to a 15 ml conical tube and centrifuged at 1400 rpm for 3 min. Supernatant was discarded and the cell pallet was treated with RBC lysis buffer for 1 min. After washing the cells with the HBSS, 10 μl of cell suspension in trypan blue were counted with a hemocytometer. Then, $1x10^6$ splenocytes were labeled with Alexa-488-CD4 (1:1000; BioLegend, San Diego, CA), PE-CD8 (1:1000; BioLegend) and APC-CD69 (1:100; BioLegend). A fraction of activated $CD4^+$, $CD8^+$ and $CD69^+$ T cells were analyzed using flow cytometry.

2.2.6. Immunohistochemistry

The removed kidneys containing transplanted islets from BALB/c nude and C57BL/6 mice were fixed in neutral 4% paraformaldehyde-phosphate-buffered saline, embedded in paraffin and sectioned at 4 μm. The tissue sections were deparaffinized in xylem and then rehydrated in serially graded alcohol. Ex-4, insulin, $CD4^+$, $CD8a^+$, $CD20^+$, and BrdU antigen retrieval consisted of heating in 10 mM citrate buffer at pH 6.0 with microwaves (5 min, 3 times, 700W), and cooling to room temperature for 20 min. After washing with distilled water, the sections were pre-incubated in 4% bovine serum albumin and dextran solution for 30 min to reduce nonspecific binding. The sections were incubated for 1 h at room temperature with mouse monoclonal anti-insulin (guinea pig α–mouse mAb; Abcam Inc., Cambridge, MA), anti-Ex-4 (rabbit α–mouse CD8a pAb; Phoenix Pharmaceuticals Inc.), anti-$CD4^+$ (mouse CD4 mAb; Abcma Inc., Cambridge, MA), anti-$CD8a^+$ (mouse α–mouse CD8a mAb; BioLegend, San Diego, CA), anti-$CD20^+$ (Goat α–mouse α-CD20 mAb; Santa Cruz, CA), and BrdU (Zymed, San Francisco, CA) at a 1:50, 1:1000, 1:100, 1:25, 1:40, 1:50 dilution in a humidified chamber, respectively. After washing, the tissue sections were observed with a peroxidase labeled polymer conjugated to secondary immunoglobulins in Tris-HCl buffer (Envision plus System-HRP labeled polyer; Dako, Glostrup, Denmark), which was incubated for 30 min at room temperature. Slides were washed, and the chromogen was developed for 5 min with liquid 3, 30-diaminbenzidine (Dako). The sections were counterstained with Mayer

hematoxylin. Negative controls were treated similarly with the exception of primary antibodies. TUNEL staining was performed using ApopTag Perodxidase *In Situ* Apoptosis detection kit (Chemicon interational, Temecula, CA).

2.2.7. Statistical analysis

The IPGTT of transplanted islets, cell viability, insulin secretion test, and apoptosis were analyzed as the mean ± SEM. The survival time, insulin level and beta cell area were analyzed as the median ± SEM. The secretion rate of Ex-4 and subpopulations of T cell analyses results were expressed as mean ± SD. The statistical analysis was carried out using the unpaired *t*-test. A *P* value of less than 0.05 was regarded as statistically significant.

2.3. Results

2.3.1. Transduction of LV-SP-Ex-4 into islets

The SP-Ex-4 gene was successfully integrated into the host DNA and encoded for the mRNA of SP-Ex-4 (data not shown). The transduction efficiency of LV-SP-Ex-4 into the islets was 7.5% as determined via FACS analysis. The Ex-4 secretion test revealed that Ex-4 was secreted from the LV-Ex-4 transduced islets at 0.15 ng/day/100 islets. On the other hand, the secretion rate of Ex-4 from the LV-SP-Ex-4 transduced islets was increased up to 0.3 ng/day/100 islets within 7 days after transduction (Fig. 2.1a).

2.3.2. Functionality and viability of LV-SP-Ex-4 transduced islets

In LV-Ex-4 transduced islets, the pattern of insulin secretion was similar to that of untransduced islets; that is, the amount of Ex-4 protein secreted from the islets was not sufficient to generate insulinotropic action in the islets. However, in the case of the LV-SP-Ex-4 transduced islets, the amount of insulin secreted in high glucose solution (28 mM) was 1.9 times elevated compared to untransduced islets (Fig. 2.1b). Consequently, the stimulation index of the LV-SP-Ex-4-transduced islet was statistically 3.0 or 2.3 fold higher than those of the untransduced or LV-Ex-4 transduced islets, respectively (Fig. 2.1c). These results demonstrated that the secretion signal peptide could profoundly enhance the secretion of Ex-4 from the islets, thereby enhancing beta cell function via the interaction between the secreted Ex-4 and GLP-1 receptor. The viabilities of LV-Ex-4 and LV-SP-Ex-4 transduced islets were measured at 88.2 ± 12.7 and 117.8 ± 4.5%, respectively, when the viability of fresh islets was adjusted to 100% (Fig. 2.2a). Additionally, the green coloring from acridine orange (AO)/propidium iodide (PI) staining indicated that the lentiviral transduction did not affect the viability of islets (Fig. 2.2b).

2.3.3. Cytokine- or hypoxia-induced apoptosis in LV-SP-Ex-4 transduced islets

The ability of Ex-4 to inhibit apoptosis during the treatment of cytokines was evaluated under *in vitro* conditions. After cytokine treatment,

the increased early apoptosis level in untransduced islets was 1.7-fold higher than that of LV-SP-Ex-4-transduced islets (Fig. 2.3a.). However, the increased caspase-3 activity of LV-SP-Ex-4 transduced islets was not statistically different from that of untransduced islets (Fig. 2.3b.). Therefore, LV-SP-Ex-4 transduction might slightly prevent cytokine-induced apoptosis in the islets. The LV-SP-Ex-4-transduced islets were found to be resistant against hypoxia-induced apoptosis. The survival rates of untransduced islets after incubation under hypoxic condition was 76.4 ± 2.3%, which was not statistically different from that of LV-SP-Ex-4 transduced islets (93.5 ± 8.9%) when the data were normalized against their respective controls (Fig. 2.3c). The caspase-3 activity of LV-SP-Ex-4 transduced islets was increased 1.3 times under hypoxic conditions relative to the normoxic condition (Fig. 2.3d). However, we noted no statistical differences in caspase-3 activity between LV-SP-Ex-4 transduced (40275 ± 4841 RLU/DNA) and untransduced islets (31156 ± 5065 RLU/DNA) under hypoxic conditions. Therefore, when compared to the untransduced islets, the tendency of LV-SP-Ex-4 transduced islets to prevent cytokine and hypoxia-induced apoptosis was not statistically significant.

2.3.4. LV-SP-Ex-4 transduced islet transplantation into diabetic nude mice

To determine whether LV-SP-Ex-4 transduction could attenuate the critical number of islets required to cure diabetes, 50 untransduced islets were

transplanted into diabetic nude mice. The non-fasting blood glucose (NBG) level of the mice fluctuated severely (Fig. 2.4a). Normally, at least 150 untransduced islets are normally required to cure the NBG level in diabetic nude mice (Fig. 2.4b); however, only 50 LV-SP-Ex-4 transduced islets were transplanted into diabetic nude mice to maintain the blood levels stably within the normal range (< 200 mg/dl) prior to nephrectomy on the 30^{th} day (Fig. 2.4c). After nephrectomy, all of the mice secreting insulin and Ex-4 became hyperglycemic, confirming the role of transplanted islets at the controlling NBG levels. Mice that received the LV-SP-Ex-4 transduced islets were unaffected by decelerated food intake and by weight gain (Fig. 2.4d). In addition, the IPGTT was conducted at day 30 of transplantation to confirm that the blood glucose level of mice with 50 LV-SP-Ex-4 transduced islets was similar to that of the normal mice (Fig. 2.4e). When the kidneys having untransduced or LV-SP-Ex-4 transduced islets were histologically analyzed on day 30 post-transplantation, only LV-SP-Ex-4 transduced islet mass secreting insulin and Ex-4 was well preserved (Fig. 2.5).

2.3.5. LV-SP-Ex-4 transduced islet transplantation into diabetic C57BL/6 mice

Diabetic immunocompetent C57BL/6 mice were implanted with 300 LV-SP-Ex-4 transduced islets and untransduced islets, respectively. The median survival time of the LV-SP-Ex-4 transduced islets was higher than untransduced islets as shown in Fig. 2.6, indicating the role of Ex-4 protein in

improving the beta cell function. At day 7 of transplantation, the beta cells mass was examined in kidney tissue containing untransduced and LV-SP-Ex-4 transduced islets slice and the total beta cell area was histologically compared. The mass area in LV-SP-Ex-4 transduced islets was 35350 ± 2330 μm^2 and that of untransduced islets was 40406 ± 7349 μm^2. The masses of both groups were not statistically different (Fig. 2.7a). In addition, the serum insulin secretion level in LV-SP-Ex-4 transduced islets transplanted group was 2.4 folds higher than untransduced islet transplanted group. (LV-SP-Ex-4: 0.66 ± 0.10 ng/ml, Control: 0.27 ± 0.05 ng/ml, *t*-teat, *P* value < 0.05) (Fig. 2.7b). The function of LV-SP-Ex-4 transduced islets was increased when compared to that of untransduced islets *in vivo*, which could improve the survival time of grafted islets. To evaluate the initiation of immune response using the LV-SP-Ex-4 transduction system, we determined the activation rates of the $CD4^+$ and $CD8^+$ T cell subpopulation in the spleen 5 days after untransduced or LV-SP-Ex-4 transduced islet transplantation. No significant populations of $CD4^+$ T cells or $CD8^+$ T cells were detected (Fig. 2.8a); in particular, no statistical differences were observed to exist between the $CD69^+CD4^+$ T cell and $CD69^+CD8^+$ T cell populations in either group ($CD69^+$: an early lymphocyte activation marker) (Fig. 2.8b). The histology data indicated that LV-SP-Ex-4 transduction to islets did not trigger any substantial immune cell activation. Thus, our local LV-SP-Ex-4 gene delivery did not have a significant effect on immune activation in the recipients. Immunohistological analysis conducted on day 7 of transplantation demonstrated that there was no significant difference in immune cell recruitment between the LV-SP-Ex-4 transduced

islets and untransduced islets. BrdU immunostaining was used to detect newly produced islet cells in LV-SP-Ex-4 transduced islets; however, no BrdU-positive cells were detected in the transplantation site. In addition, TUNEL positive cells in the control islet transplanted group existed around the transplanted site. On the other hand, no TUNEL-positive cells were observed in section from LV-SP-Ex-4 transduced islet (Fig. 2.9). Collectively, the LV-SP-Ex-4 transduction itself could not initiate the immune response, but it positively enhanced the beta cell function of the transplanted islets. Furthermore, LV-SP-Ex-4 transduction system could efficiently inhibit cell apoptosis in islets transplanted *in vivo*.

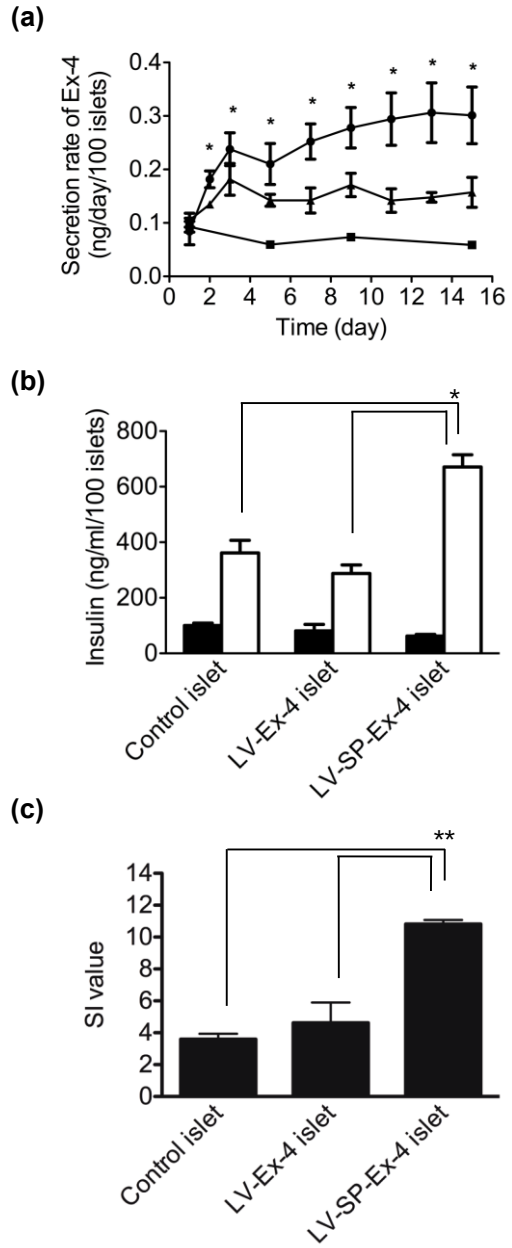

Figure 2.1. (a) The secretion rate of Ex-4 from LV-SP-Ex-4 (black circle) and LV-Ex-4 (black triangle) transduced and untransduced (black square) islets for 15 days *in vitro*. Data were expressed as mean ± SD (n=5) (b) The glucose stimulated-insulin secretion of untransduced (control), LV-Ex-4 transduced or

LV-SP-Ex-4 transduced islets in low (black bar, 2.8 mM) or high (white bar, 28 mM) glucose solutions for 1 h, respectively (c) SI value of untransduced (control), LV-Ex-4 transduced or LV-SP-Ex-4 transduced islets. Data were expressed as mean ± SEM (n=5), *$P < 0.05$ and **$P < 0.001$ compared with the control and LV-Ex-4 islet groups.

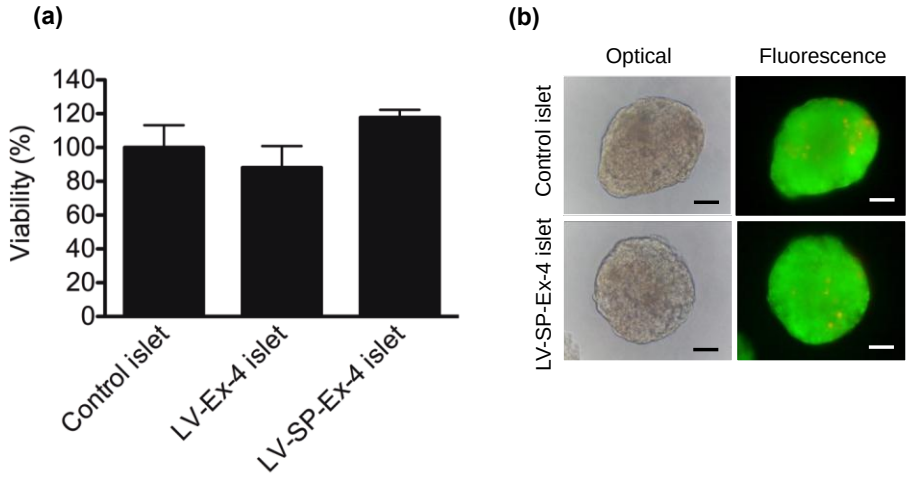

Figure 2.2. (a) The viability of untransduced (control), LV-Ex-4 transduced and LV-SP-Ex-4 transduced islets using CCK-8 assay (b) The fluorescence images of untransduced and LV-SP-Ex-4 transduced islets visualized via AO/PI stain method. The morphology and fluorescence were observed and photographed using a fluorescence microscope on day 3 post-transduction. Data were expressed as mean ± SEM (n=4), Scale bar = 50 μm

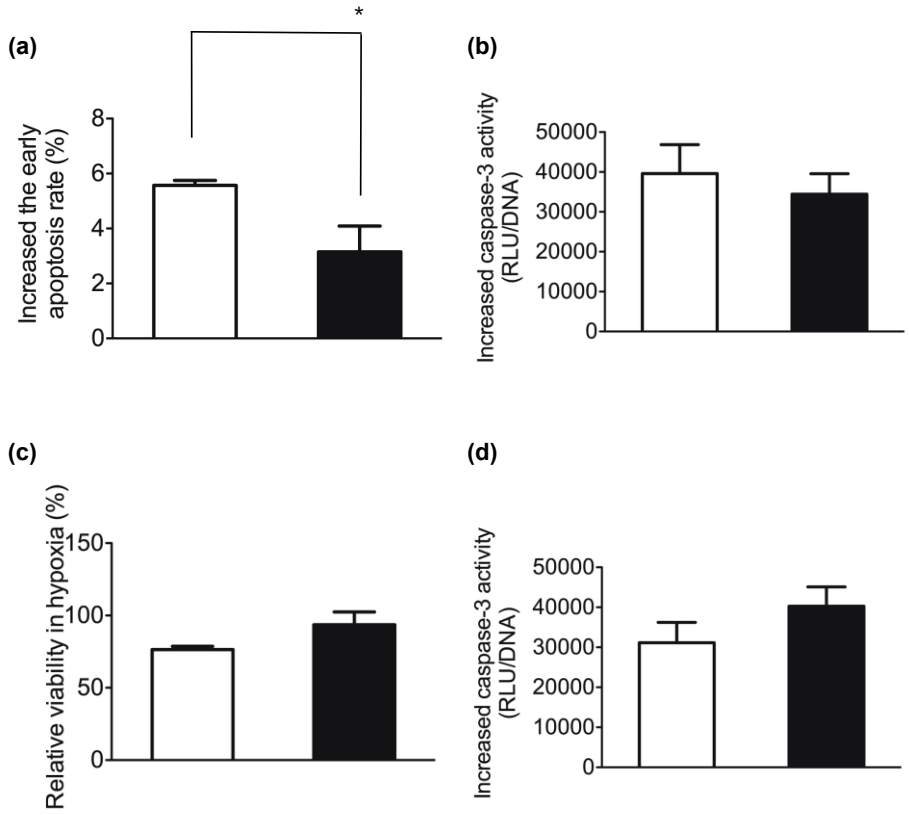

Figure 2.3. Relative cell viability and anti-apoptotic effect of LV-SP-Ex-4 transduced islets. (a) and (b) increased early apoptosis percentage and caspase-3 activity of untransduced (control; white bar) and LV-SP-Ex-4 transduced (black bar) islets after cytokine treatment (IL-1β; 100 U/ml, TNF-α; 1000 U/ml, IFN-γ; 1000 U/ml) for 3 days (n=4). (c) and (d): The viability and increased caspase-3 activity of untransduced (control; white bar) islets and LV-SP-Ex-4 transduced (black bar) after 24 h of incubation under hypoxic conditions. Data were expressed as mean ± SEM (n=4). *$P < 0.05$.

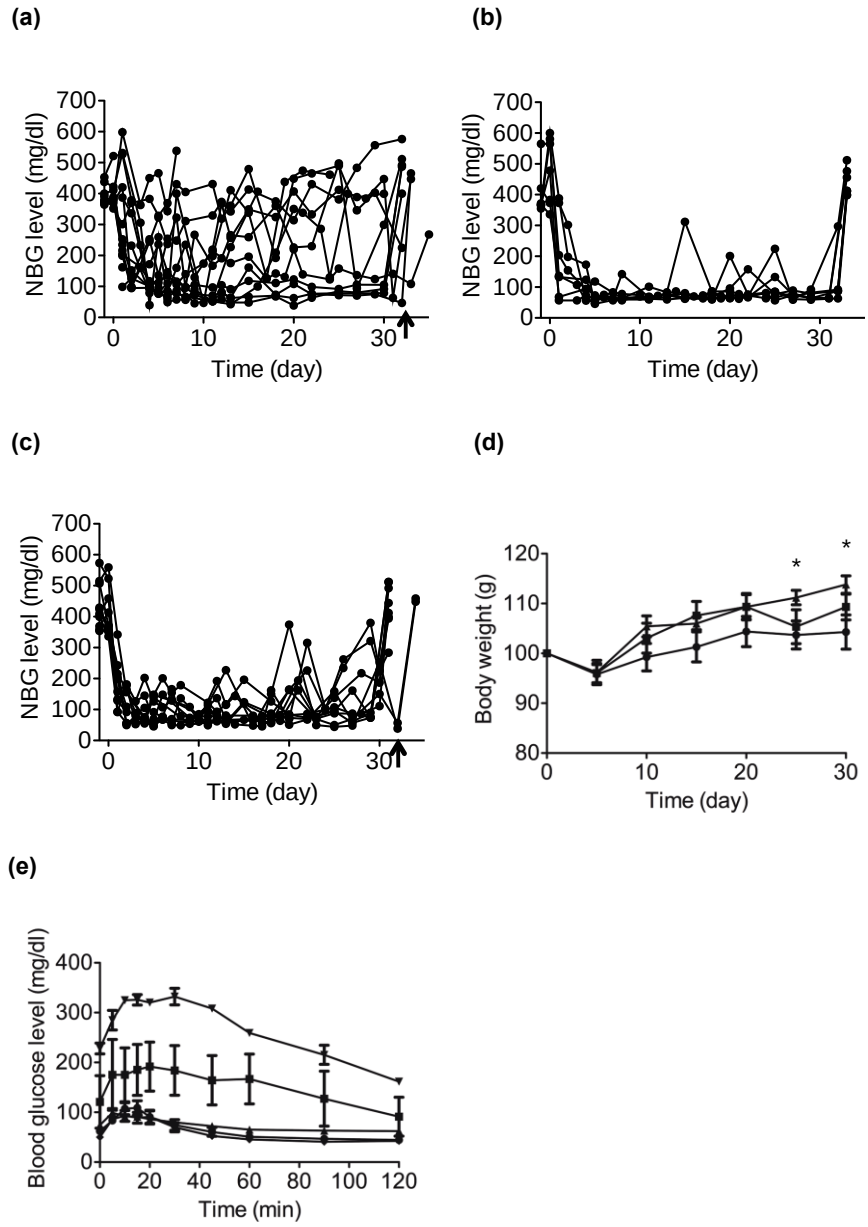

Figure 2.4. Non-fasting blood glucose levels after islet transplantation into STZ-induced diabetic nude mice. (a) The blood glucose levels of mice harboring 50 untransduced islets (n=13). (b) Blood glucose levels of mice having 150 untransduced islets (n=7). (c) Blood glucose levels of mice having 50 LV-SP-Ex-4 transduced islets (n=13). (d) The body weights of the

recipients receiving 50 LV-SP-Ex-4 transduced islets (black triangle, n=13), 150 untransduced islets (black square, n=7), 50 untransduced islets (black circle, n=13). (Mice which received 50 untransduced islets vs. mice which received 50 LV-SP-Ex-4 transduced islet, $*P < 0.05$). Data were expressed as mean ± SEM (e) The IPGTT of normal (black triangle), diabetic (black reverse triangle), 50 untransduced islets transplantation (black square), 150 untransduced islets (black diamond), 50 LV-SP-Ex-4 transduced islets (black circle), taken30 days after transplantation. Data were expressed as mean ± SEM (n=5). Arrow: Nephrectomy surgery after 30 days of transplantation.

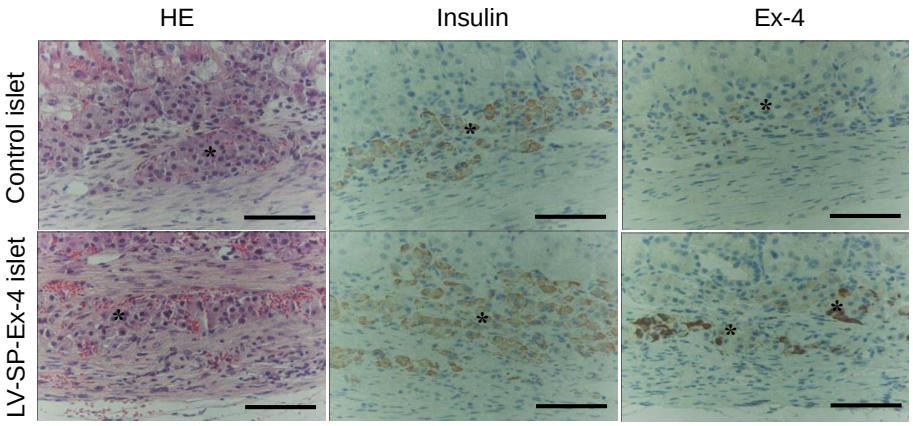

Figure 2.5. Anti-insulin and anti-Ex-4 immunostains of transplanted islets shown 30 days after islet transplantation; Ex-4 positive cells were observed abundantly in LV-SP-Ex-4 transduced islet-bearing kidneys. Asterisk: transplanted islets, Scale bar = 100 μm.

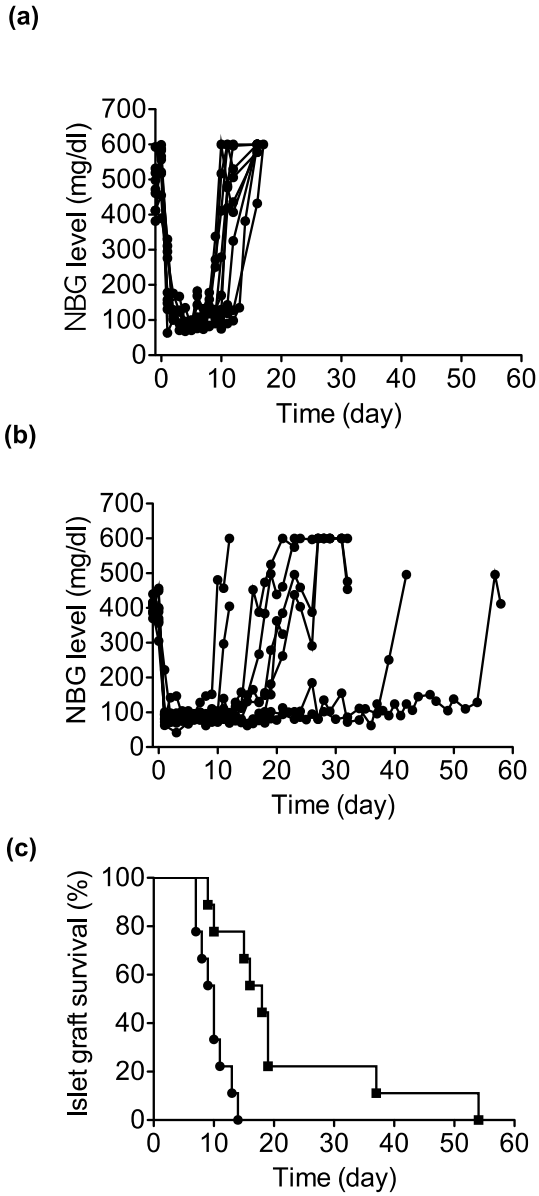

Figure 2.6. Non-fasting blood glucose levels after islet transplantation into immunocompetent STZ-induced diabetic C57BL/6 mice. (a) 300 untransduced islet-transplanted group (n=9), (b) 300 LV-SP-Ex-4 transduced islet-transplanted group (n=9), (c) Survival graft rate of LV-SP-Ex-4 transduced (black square) and untransduced (black circle) islets.

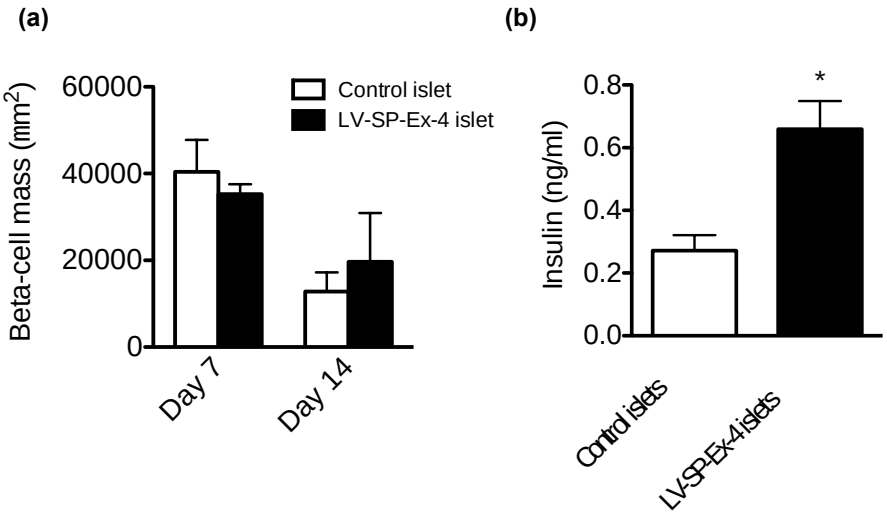

Figure 2.7. Serum insulin level in islets transplanted C57BL/6 mice after 14 days of transplantation and beta cell mass of transplanted islets at day 7 of transplantation (a) Insulin level and (b) beta cell mass. Data were expressed as mean ± SEM (n=3), *$P < 0.05$

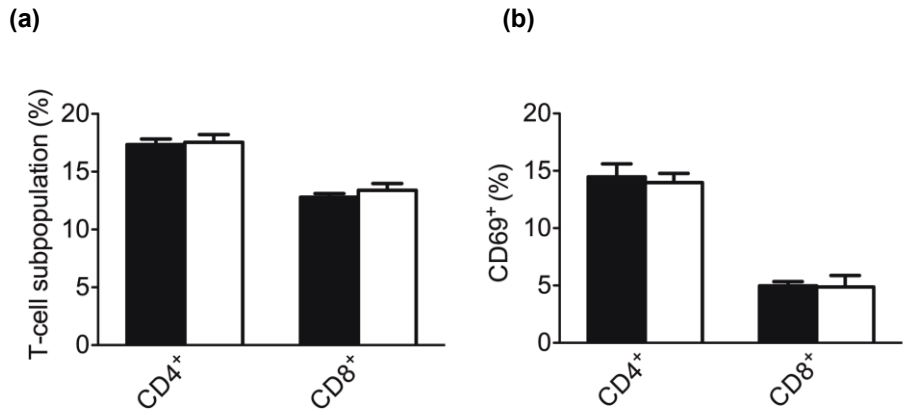

Figure 2.8. (a) T-cell subpopulations and (b) early T cell activation in the spleen tissues of C57BL/6 mice harboring untransduced islets (black bars) or LV-SP-Ex-4 transduced islets (white bars) at 5 days after transplantation. Data were expressed as mean ± SD. (n=3)

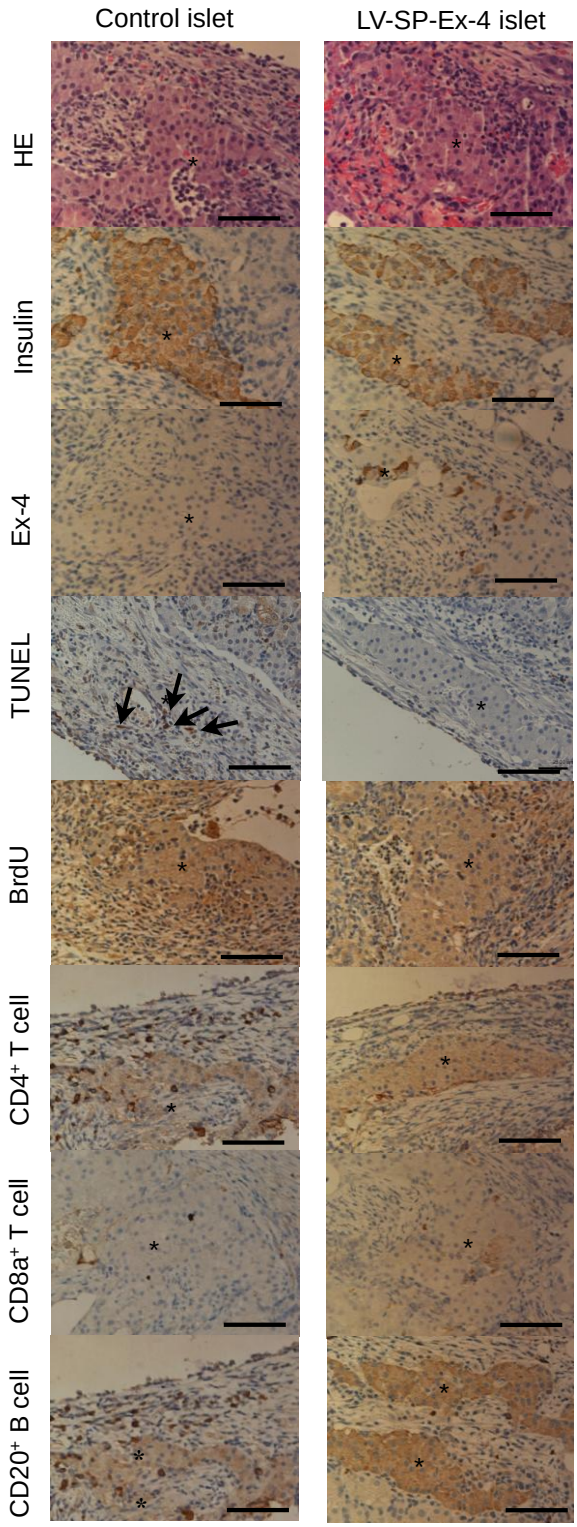
Control islet
LV-SP-Ex-4 islet
HE
Insulin
Ex-4
TUNEL
BrdU
CD4+ T cell
CD8a+ T cell
CD20+ B cell

Figure 2.9. Anti-insulin, anti-exendin-4, anti-$CD4^+$, anti $CD8a^+$, anti $CD20^+$, anti-BrdU and TUNEL immunostaining of transplanted islets at day 7 of transplantation. Asterisk: transplanted islets, Scale bar = 100 μm

2.4. Discussion

In this study, we designed a novel system that facilitates the release of abundant quantities of Ex-4 from the transplanted islets for its local action. The transduction efficiency of LV-SP-Ex-4 in islets (7.4%) was low due to the collagen layer covering the islet surface that prevents the insertion of lentivirus into the islet cells. Thus, only the external areas of the islets were transduced with LV-SP-Ex-4. Nevertheless, the Ex-4 was continuously secreted from the LV-SP-Ex-4 transduced islets and could thus effectively carry out insulinotropic activities in the islets under *in vitro* and *in vivo* conditions. This was because the insertion of a SP sequence into the vector profoundly enhanced the secretion of Ex-4 from the LV-SP-Ex-4 transduced islets. Additionally, the secreted Ex-4 did not contain the secretion signal peptide fragment because the Furin cleavage site (RGRR) is inserted between the SP and Ex-4 genes. This enabled Ex-4 secreted from the genetically engineered islets to interact properly with the GLP-1 receptor and effectively reduce the number of islets required to control the blood glucose of diabetic mice within a normal range.

We determined that only 50 LV-SP-Ex-4 transduced islets were required to cure diabetes in a single mouse due to the insulinotropic action of the Ex-4 secreted from the islets. In the case of untransduced islet transplantation, at least 150 islets had to be transplanted into a diabetic nude mouse to achieve normoglycemia. In addition, we observed a better outcome of prolonged islet survival time in the immunocompetent diabetic C57BL/6

mice into which LV-SP-Ex-4 transduced islets were transplanted. At day 14 of transplantation, the serum insulin level of LV-SP-Ex-4 transduced islets transplanted recipients was significantly increased when compared to that of untransduced islets transplanted recipients. Exendin-4 protein secreted from LV-SP-Ex-4 transduced islets effectively increased the function of islets. Therefore, these results show that the essential number of islets for curing diabetes might be reduced significantly, effectively overcoming the shortage of islet donors.

To demonstrate the anti-apoptotic action of the Ex-4 against immune cells, the LV-SP-Ex-4 transduced islets were treated with cytokines and incubated under hypoxic conditions, respectively. The results demonstrated that the secreted Ex-4 could not inhibit cytokine- and hypoxia-induced apoptosis in the transduced islets. The reason for this unremarkable inhibition of cytokine-induced apoptosis might be that the amount of secreted Ex-4 protein was not sufficient to elicit a complete therapeutic action and that the condition of cytokine-induced apoptosis to islets *in vitro* was much higher than that observed under *in vivo* conditions. However, Ex-4 protein secreted from LV-SP-Ex-4 transduced islets sufficiently inhibited the apoptosis *in vivo*, which was evaluated by TUNEL assay [13, 22, 23]. Collectively, locally delivered LV-SP-Ex-4 gene delivery system effectively inhibited the apoptosis *in vivo* although they did not exhibit an anti-apoptotic effect in hypoxi- and cytokine-induced apoptotic condition *in vitro*. Even though it was reported that Ex-4 would play an important role in beta cell proliferation [24, 25], the BrdU incorporation staining in this study revealed no beta cell proliferation in

either the LV-SP-Ex-4 transduced islets or untransduced islets. In addition, Baggio et al. have reported a severe lymphocytic infiltration and immune activation with increased numbers of immune cells in the liver and kidney in metallothionein promoter-exendin-4 (MT-Exendin) transgenic mice [26]. Thus, we confirmed activation of the immune system after LV-SP-Ex-4 gene transduction system by measuring the T cell population after LV-SP-Ex-4 transduced islet transplantation. The activation of $CD4^+$, $CD8^+$, $CD69^+CD4^+$, $CD69^+CD8^+$ T cell populations in the spleen tissues of the mice were detected 5 days after islet transplantation, which confirms immune activation in mice with LV-SP-Ex-4 transduced islets. No statistically significant differences were noted to exist between LV-SP-Ex-4 transduced islet transplantation and untransduced islet transplantation. Local immune cell activation was similarly detected in both LV-SP-Ex-4 transduced and untransduced islets, thereby suggesting that there was no evidence of a significantly greater cumulative incidence of lymphocyte infiltration in and/or around LV-SP-Ex-4 transduced islets relative to that observed in the untransduced islets.

This study highlights the potent insulinotropic action of LV-SP-Ex-4 transduction on islets as a major mechanism for enhancing insulin secretion. This genetic engineering of islets can contribute profoundly to the efficiency of transplanted islets by reducing the number of transplanted islets required in the mouse model. Additionally, as compared to the systemic injection of the Ex-4, this new technology can attenuate the adverse effects of Ex-4, as it can be locally secreted at the site of islet transplantation. Therefore, the highly releasable LV-SP-Ex-4 transduction system described herein may help to

explain the observed enhancements of the beta cell function in islet transplantation.

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Chapter 3. Surface Camouflage of Pancreatic Islets Using 6-arm-PEG-catechol in Combined Therapy with Tacrolimus and Anti-CD154 Monoclonal Antibody for Xenotransplantation

3.1. Introduction

Pancreatic islet transplantation is a promising method to treat type 1 diabetes, albeit transplanted islets are susceptible to rejection by the immune reaction. To protect the transplanted islets against immune reactions, several kinds of immunosuppressant drugs such as tacrolimus and sirolimus should be administered. In this regard, the 'Edmonton protocol' has been accepted as one of the best protocols outlined for clinical islet transplantation [1, 2]. However, a long-term immunosuppressive therapy is accompanied by several adverse effects such as nephrotoxicity, neurotoxicity, infectious disease, hypertension, etc. [3-5]. In addition, Shapiro et al. reported that only below 10% of patients under the Edmonton protocol achieved insulin independence after 5 years of transplantation [1]. Thus, a new immunosuppressive remedy has been in need to enhance the immunoprotection efficacy with reducing adverse effects of immunosuppressive drugs.

As a method for preventing host immune cell infiltration into transplanted islets, microencapsulation of islets has been proposed. However, the relatively large size of microcapsules influenced the consequential limitation of mass and oxygen transports, and the size of microencapsulated islets was not suitable for transplantation via hepatic portal vein. Also, alginate, a general material for microencapsulation of islets, was known to induce the inflammation because of its impurity [6, 7]. On the other hand, the surface camouflage of islets using poly(ethylene glycol) (PEG) [8-11], heparin [12, 13] and chitosan derivatives [13] have been proposed as

promising methods for preventing immune responses. Cabric et al. reported that modification of islet surface using heparin protected the islet acute immune reaction by innate immune system in an intraportal islet transplantation [12, 14]. Teramura et al. reported surface modified islet using poly(ethylene glycol)-phospholipid conjugates (PEG-lipid) for improvement of graft survival in an intraportal transplantation. It was reported that the survival time of PEG-lipid grafted islets was slightly longer than the control islets when the recipient was transplanted with 500 islets (PEG-lipid grafted islets; 5.0 ± 0.9 days, control islets; 1.8 ± 1.6 days, $P<0.01$); however, the survival time of grafted islets was not sufficiently increased [15]. Therefore, polymeric therapy like a surface modification strategy did not perfectly protect the host immune reaction and cytokine attack activated by immune system. We have reported on the limitation of surface camouflage of islets for protection against immune reactions and improved the survival graft time of monomethoxy-poly(ethylene glycol)-succinimidyl propionate (mPEG-SPA) grafted islets with the treatment of cyclosporin A in allotransplantation [16].

In this study, we assumed that the catechol moiety anchored 6-arm-PEG (6-arm-PEG-catechol) could be more highly packed on the islet surface compared to the linear PEG. Hence we propose that it could effectively prevent immune cell infiltration and activation in xenotransplantation. In addition, catechol moieties, extracted from adhesive protein of mussel, were conjugated to the functional end group of 6-arm-PEG in order to effectively conjugate PEG molecules to the islet surface. Furthermore, to evaluate the ability of applying this surface camouflage system to clinical study, we

assume that it would be possible to expand the synergistic effect if we use this 6-arm-PEG-catechol grafting technique in combination with low doses of the immunosuppressive drugs. In this study, a low dose of tacrolimus (FK506), a calcineurin inhibitor, was used to prevent generation of cytokines from different immune cell activation. Anti-CD154 mAb (MR-1) was also used to inhibit the activation of $CD4^+$ T cell that is triggered by indirect immune activation pathway in xenotransplantation. Here, we suggested a convergent protocol using surface camouflage technique and low doses of immunosuppressive drugs to improve islet xenograft survival rate.

3.2. Materials and Methods

3.2.1. Synthesis scheme of 6-arm-PEG-catechol

Six-arm-PEG-amine (1 g, mw 15 kDa) was dissolved in 10 ml of N-methylpyrrolidone (NMP) at 60˚C for 10 min. DHCA (3,4-dihydroxyhydrocinnamic acid, 0.8 mmol), benzotriazole-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate (BOP, 0.8 mmol), 1-hydroxybenzotriazole (HOBt, 0.8 mmol) and N,N-diisopropylethylamine (DIPEA, 0.8 mmol) were dissolved in 5 ml of NMP in a separate vials. First, both PEG and DHCA solutions were reacted at room temperature for 6 h, followed by reacting with BOP, HOBt and DIPEA until Ninhydrin assay showed a negative result. The reacted solution was dialyzed (MWCO 8 kDa) in acidified distilled water (pH = 1~2) to prevent catechol oxidation, followed by lyophilizing.

3.2.2. Animals

Inbred male C57BL/6 mice (7~8 weeks of age) were used as recipients and male Sprague-Dawley (SD) rats (8 weeks of age) were used as donors. They were purchased from Orient Bio Inc. (Seongnam, South Korea) and were housed under a specific pathogen-free condition. Diabetes mellitus was chemically induced in C57BL/6 mice by a single intraperitoneal injection of 180 mg/kg of streptozocin (STZ) (Sigma, St. Louis, MO). Mice, which exhibited the glucose level over 300 mg/dl for two consecutive days, were selected as diabetic recipients for transplantation. All experimental and surgical procedures were carried out according to the guidelines of the Institute of Laboratory Animal Resources, Seoul National University (IACUC no. SNU-070822-5)

3.2.3. Islet isolation and 6-arm-PEG-catechol grafting

Pancreatic islets were isolated from outbred male SD rats by digestion of the pancreas using collagenase (Sigma, St. Louise, MO) and purification using discontinuous Ficoll™ PM400 (Amersham Biosciences AB, Uppsala, Sweden) density gradient centrifugation. Isolated islets were then cultured for 2 days in the RPMI-1640 culture medium (Sigma) containing 10% fetal bovine serum (FBS; Sigma) at 37℃ under the humidified atmosphere containing 5% CO_2. Firstly, the isolated islets were washed twice with the HBSS (pH 8.0), and then 6-arm-PEG-catechol was grafted onto the islet surface. The 6-arm-PEG-catechol grafted islets were then suspended in 10 ml

of HBSS (pH 8.0) containing 25 mg, 100 mg and 500 mg of 6-arm-PEG-catechol (0.25, 1 and 5%, w/v).

3.2.4. Cell viability

Viability of islets was qualitatively analyzed by Live/Dead Viability/Cytotoxicity Kit assay (Molecular Probes, Eugene, OR). Since the activity of intracellular esterase causes non-fluorescent cell-permeable calcein AM to become intensely fluorescent, the viable islets then produce an intense uniform green fluorescence. Ethidium homodimer (EthD-1) enter into the damaged islet membrane and then bind to nucleic acids, thereby producing a red fluorescence in the dead islets. Islets were cultured in the RPMI-1640 culture medium containing 10% fetal bovine serum for 3 days at 37℃ under the humidified atmosphere containing 5% CO_2. These islets were washed twice with HBSS, followed by suspending in 1 ml of HBSS containing 2 μl of 50 μM calcein AM working solution and 4 μl of the 2 mM EthD-1 for 15 min at room temperature. The islets stained with calcein AM and EthD-1 were observed under light microscope (Eclipse TE2000-S, Nikon, Japan).

The viability of islets was also quantitatively analyzed by Cell Counting Kit-8 (CCK-8) assay. The suspended islets (50 islets/well) were dispensed into a 96-well culture plate and treated with 10 μl of WST-8 [2-(2-methoxy-4-nitorphenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrozoium, monosodium salt]. The islets treated with WST-8 solution were incubated for 2.5 h and the absorbance was measured at 450 nm using a

microplate reader. The viability of islets was also measured by the oxygen consumption rate (OCR) as follows: four hundreds islets were suspended in HBSS and 200 μl of suspended solution was added into a stirred titanium chamber (MicroOxygen Uptake System FO/SYSZ-P250, Plymouth meeting, Instech Laboratories, PA) at 37℃. Oxygen partial pressure was recorded with a fluorescence based oxygen sensor (Neofox; Ocean Optics, FL) with time. The OCR data was normalized by DNA contents of each chamber.

3.2.5. Six-arm-PEG-catechol grafting onto the islet surface

Fluorescein isothiocyanate (FITC) linked 6-arm-PEG-catechol was immobilized to evaluate the distribution of 6-arm-PEG-catechol on the surface of islets. The molecule of FITC was reacted with 20% of amine residue in 6-arm-PEG-amine and the remaining amine residue was saturated by catechols. The immobilization of FITC-linked 6-arm-PEG-catechol was prepared by suspending freshly isolated islets in HBSS (pH 8.0) solution containing FITC-linked 6-arm-PEG-catechol (0.25%, w/v). FITC intensity was measured using a laser scanning confocal microscope (LSM510, Carl Zeiss, Germany).

3.2.6. Glucose-stimulated insulin secretion (GSIS) assay

Six-arm-PEG-catechol modified and unmodified islets were suspended in Krebs-Ringer bicarbonate buffer (KRBB; pH 7.4) containing 2.8 mM glucose, respectively. Then, 100 islets were plated into a 24-well culture

plate containing a millicell culture plate insert. After pre-culture at 37℃ for 1 h under the humidified atmosphere containing 5% CO_2, the medium was exchanged with the same buffer to measure glucose stimulated insulin secretion during 2 h incubation in KRBB containing a low glucose solution (2.8 mM). Then, the medium was exchanged again with KRBB containing a high glucose solution (28 mM) for 2 h. After incubation of the islets, the secreted amount of insulin from each samples were measured using a rat/mouse insulin ELISA kit (Millipore, MA). The stimulation index (SI) value was calculated by dividing the amount of insulin secreted at a high glucose solution by that at a low glucose solution.

3.2.7. Xenotransplantation of rat islets into diabetic mice

Chemically induced diabetic C57BL/6 mice were anaesthetized by intraperitoneal injection with ketamin 80 mg/kg and xylazine 16 mg/kg. The left kidney of the recipient was exposed thorough the lumbar incision. Six-arm-PEG-catechol modified islets and unmodified islets (300 IEQ) were transplanted on the left kidney capsule. After islet transplantation, both body weight and non-fasting blood glucose concentrations were monitored from tail veins of mice using a portable glucometer (Super glucocard II, Arkray, Kyoto, Japan). The islet transplantation was considered a success if the blood glucose level was lower than 200 mg/dl for two consecutive days, and the transplanted islets were considered as rejected if the blood glucose concentration was higher than 200 mg/dl for two consecutive days.

In addition, intraperitoneal glucose tolerance test (IPGTT) using 20% glucose solution (Sigma) was performed to evaluate the glucose responsiveness of the transplanted islets at day 50 of transplantation. Examined groups were fasted at least for 6 h before the experiment with free access to water prior to the glucose tolerance test. A solution of 20% D-glucose (10 µl/g) was injected into the peritoneal cavity and the change of the blood glucose was measured from the tail vein at different time interval.

Synergistic effects of Tacrolimus (FK506; Prograf®, Astellas Pharmaceuticals, Chicago, IL), MR-1 (anti-CD154 mAb, BioXcell, West Lebanon, NH) and PEGylation on immunoprotection of transplanted islets were evaluated as follows: Recipients were daily administered intraperitoneally 0.2 mg/kg of Tacrolimus diluted with 200 µl PBS (pH 7.4). Separately, anti-CD154 mAb (0.1 mg/mouse) was intraperitoneally injected into recipients on 0, 2, 4, and 6 days of post-transplantation.

3.2.8. Immunohistochemistry

The left kidney containing the transplanted islets was retrieved, and it was fixed in neutral 4% paraformaldehyde-phosphate-buffered saline, embedded in paraffin and sectioned at 4 µm. The tissue sections were deparaffinized in xylene and then rehydrated in serially graded alcohol. Insulin antigen retrieval consisted of heating in 10 mM citrate buffer of pH 6.0 by microwaves (5 min, 3 times, 700 W), and then cooling to room temperature for 20 min. After washing with distilled water, the slides were

preincubated in 4% bovine serum albumin and dextran solution for 30 min to reduce nonspecific binding. The slides were incubated for 1 h at room temperature with mouse monoclonal anti-insulin (Abcam Inc., Cambridge, MA), anti-$CD20^+$ (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-$CD4^+$ (Abcam Inc.), and anti-$CD8a^+$ (BioLegend) at a 1:50, 1:40, 1:1000 and 1:100 dilution in a humidified chamber, respectively. After washing, the tissue sections were observed with a peroxidase labeled polymer conjugated to goat anti-mouse immunoglobulins in Tris-HCl buffer (Envision plus System-HRP labeled polyer; Dako, Glostrup, Denmark), which was incubated for 30 min at room temperature. Slides were washed, and the chromogen was developed for 5 min with liquid 3,30-diaminbenzidine (Dako). The slides were counterstained with Mayer hematoxylin. Negative controls were treated similarly with the exception of primary antibodies.

3.2.9. Statistical analysis

Survival time of transplanted islets were analyzed as median SEM. Cell viability and insulin secretion test analysis were expressed as mean ±S.D. Statistically analysis was carried out using the unpaired t-test or ANOVA one-way test. A p value of less than 0.05 was considered to be statistically significant.

3.3. Results

3.3.1. Characterization of 6-arm-PEG-catechol grafted islets

Six-arm-PEG-catechol was prepared by HOBt/BOP coupling reaction between the amine groups of 6-arm-PEG-amine and the carboxylic group of 3,4-dihydroxyhydrocinnamic acid. The reaction was monitored by Ninhydrin test, indicating that all amine groups of 6-arm-PEG molecule were reacted with 3,4-dihydroxyhydrocinnamic acid. FITC linked 6-arm-PEG-catechol was also prepared to visualize surface modifications of islets. By Ninhydrin test and 3,4-dyhydroxyhydrocinnamic standards (A_{280}), about 20% amine groups of 6-arm-PEG-amine were coupled with FITC. The reaction between 6-arm-PEG-catechol and collagen matrix of the islet surface was illustrated in Fig. 3.1. The cytotoxicity of 6-arm-PEG-catechol against islets was visually evaluated using a LIVE/DEAD Viability/Cytotoxicity Kit. The endocrine cells composing the outer layer of islets were visibly dead when they were suspended in the HBSS solution containing 1 and 5% of 6-arm-PEG-catchol. Cell viability of 1% 6-arm-PEG-catechol grafted islets was 81.2 ± 21.4% when measured using CCK-8. However, islets grafted with 0.25% of FITC labeled 6-arm-PEG-catechol showed green fluorescence even at the outer layer of surface (Fig. 3.2a,b). Most of the single islet cells were alive after the 6-arm-PEG-catechol immobilization. The PEG incubation time was optimized as 1 h from our previous study [17]. One-hour incubation time was sufficient to cover the whole surface of islets. Therefore, for optimal PEGylation condition, the concentration of 6-arm-PEG-catechol was set at

0.25% and incubated for 1 h incubation.

The viability and functionality tests were performed using 0.25% 6-arm-PEG-catehol grafted islets. Both Cell Counting Kit-8 assay and OCR/DNA assay demonstrated that the cell viability of 6-arm-PEG-catechol grafted islets were 105.0 ± 17.2% and 101.8 ± 3.1%, compared to that of unmodified islets, respectively (Fig. 3.2b, c). There was no statistical difference in the cell viability between 6-arm-PEG-catechol grafted islets and unmodified islets. Therefore, it was confirmed that the surface camouflage using 6-arm-PEG-catechol did not affect the viability of islets.

To confirm the degree of cell coverage, the distribution profile of FITC-linked 6-arm-PEG-catechol grafted islets was observed under confocal laser scanning microscopy (CLSM). As shown in Fig. 3.3a, 6-arm-PEG-catechol was mostly grafted on the surface of islets. The optimized PEG concentration and incubation time prevented the diffusion of PEG molecule inside the islets. The fluorescence intensity of projection image indicated that the FITC-linked 6-arm-PEG-catechol was evenly distributed to the whole surface of islets (Fig. 3.3b). However, the surface of islets was not smooth and some PEG aggregates were found on the surface and this is due to the reactivity of catechol moieties unbound to the collagen matrix of islets. They reacted with adjacent catechol moieties and formed aggregates on the surface of islets.

3.3.2. Glucose-stimulated insulin secretion test of 6-arm-PEG-catechol grafted islets

The glucose-stimulated insulin secretion was measured to evaluate the ability of 6-arm-PEG-catechol grafted islets to control the insulin release in response to glucose. There was no significant difference in an insulin release between unmodified islets and 6-arm-PEG-catechol grafted islets. At a high glucose concentration, the secretion rates of insulin from 50 unmodified islets and 50 6-arm-PEG-catechol grafted islets were 37.29 ± 17.55 ng/h and 34.50 ± 19.28 ng/h, respectively (Fig. 3.4a). The stimulation index (SI) values of unmodified islets and 6-arm-PEG-catechol grafted islets were 4.4 ± 1.1 and 6.1 ± 1.4, respectively (Fig. 3.4b). These SI values were not statistically different, and this result indicated that the grafted 6-arm-PEG-catechol did not affect the functionality of islets for releasing insulin.

3.3.3. Islet transplantation in diabetic mice

To evaluate the therapeutic potential of 6-arm-PEG-catechol grafted islets, the islets were transplanted under the left kidney capsule of chemically induced (STZ) diabetic mice, followed by measuring non-fasting blood glucose levels of recipients (Fig. 3.5). The median survival times (MST) of unmodified islet and 6-arm-PEG-catechol grafted islets were 10.5 ± 1.3 days and 12.0 ± 1.1 days (median ± SEM, $p<0.05$), respectively. Although the MST of 6-arm-PEG-catechol grafted islets was slightly increased compared to that of unmodified islets, all the transplanted islets were rejected within 20 days.

To verify the synergistic effect of surface camouflage using 6-arm-PEG-catechol and tacrolimus, 0.2 mg/kg of tacrolimus was daily administrated after 6-arm-PEG-catechol grafted islets or unmodified islets were transplanted. tacrolimus did not improve the MST of unmodified islets, and the islets in all of the mice were completely rejected within 2 weeks (MST: 10.0 ± 2.9 days). However, when tacrolimus was daily administered after 6-arm-PEG-catechol grafted islets were transplanted, MST of the islets was increased up to 21.0 ± 1.9 days. Thus, the results of the statistical analysis ($p<0.01$) show that the treatment of tacrolimus for 6-arm-PEG-catechol grafted islets could increase the survival time of islets unlike the unmodified islets in xenotransplantation.

On day 15 of transplantation, the left kidney containing transplanted islets was nephrectomized and immunostained to analyze the secretion of insulin and recruitment of immune cells (Fig. 3.6). In the case of unmodified islets, most of islets disappeared, and a little amount of secreted insulin was detected. In contrast, many $CD4^+$, $CD8^+$ and $CD20^+$ positive immune cells were detected at the graft transplanted site. On the other hand, tissue containing 6-arm-PEG-catechol grafted islets had higher amounts of islet cell mass and insulin than the control. $CD8^+$ and $CD20^+$ positive immune cells were rarely detected around the transplanted site. However, $CD4^+$ T cells were still detected near the transplanted site, explaining that the grafted 6-arm-PEG-catechol could not attenuate the T cell activation. Therefore, $CD4^+$ T cells might be the main reason why tacrolimus could not significantly prolong the survival time of 6-arm-PEG-catechol grafted islets in xenotransplantation.

To prevent the $CD4^+$ T cell activation, which is triggered by migrated antigen

from transplanted islets, anti-CD154 mAb was additionally administered (Fig. 3.7). anti-CD154 mAb could increase the survival time of transplanted islets in each case of unmodified islets and 6-arm-PEG-catechol grafted islets. Both anti-CD154 mAb and tacrolimus further increased the survival time of transplanted islets. Upon injection of anti-CD154 mAb and tacrolimus, all recipients receiving 6-arm-PEG-catechol grafted islets maintained the normal glucose level until nephrectomy at day 50 of transplantation. When 6-arm-PEG-catechol grafted islets were treated with anti-CD154 mAb and tacrolimus, the blood glucose level was more stably maintained in the normal range without any fluctuation although the MST of these islets was not statistically different from that of the unmodified islets.

As shown in Fig. 3.8, large mass of insulin positive cells was detected, and $CD4^+$ and $CD8^+$ T cells and $CD20^+$ B cells were rarely detected around the transplant site when 6-arm-PEG-catechol grafted islets were transplanted with administering anti-CD154 mAb and tacrolimus.

IPGTT confirmed glucose responsiveness of the 6-arm-PEG-catechol grafted islets treated with tacrolimus and anti-CD154 mAb at day 50 of transplantation (Fig. 3.9). After a high dose of glucose was administered to the normal mice, the blood glucose level was slightly increased and rapidly returned to the normal range within 2 hours. On the other hand, the blood glucose level of diabetic mice remained greater than 500 mg/dl after 15 min of the glucose injection. In the case of 6-arm-PEG-catechol grafted islets, the blood glucose level profile was similar to that of normal mice, indicating that 6-arm-PEG-catechol grafted islet recipients treated with tacrolimus and anti-

CD154 mAb had a normal glucose sensitivity until day 50 of transplantation.

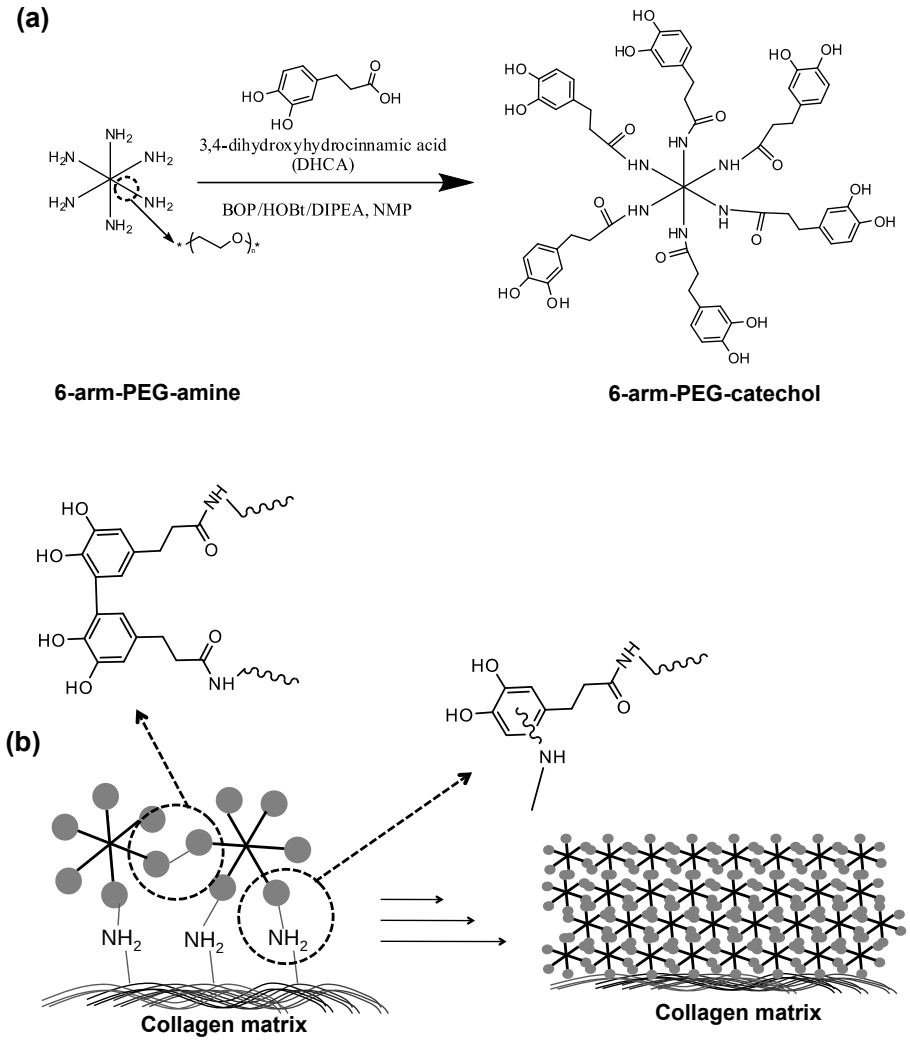

Figure 3.1. (a) Synthesis of 6-arm-PEG-catechol from 6-arm-PEG-amine. (b) Schematic representation illustrating the interaction between 6-arm-PEG-catechol and collagen matrix of the islet surface.

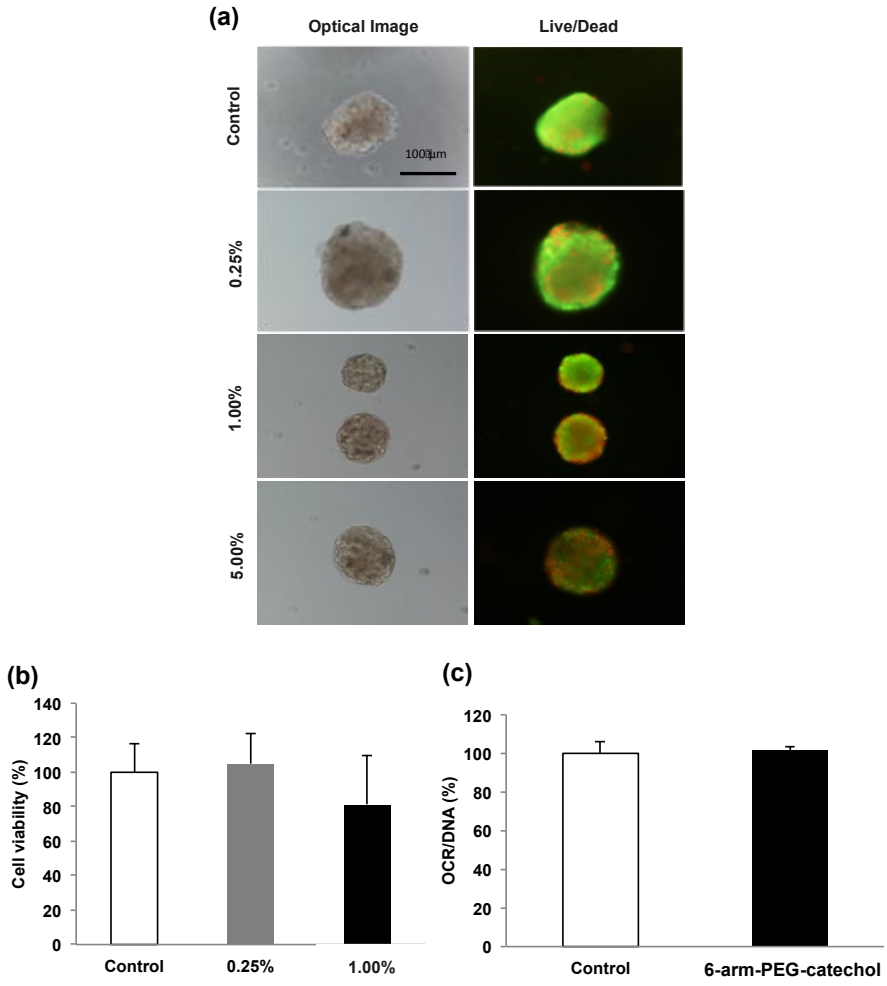

Figure 3.2. (a) Live/dead cell image of unmodified (control) and 6-arm-PEG-catechol grafted islets (0.25%, 1%, and 5%) (b) The relative viability of unmodified and 6-arm-PEG-catechol grafted islets (0.25%, 1%) using CCK-8 assay (n=4). (c) OCR/DNA value of unmodified and 6-arm-PEG-catechol grafted islets (0.25%) (n=3).

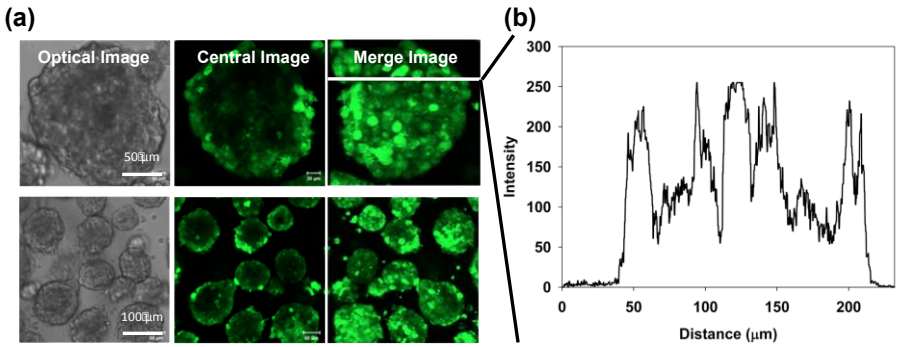

Figure 3.3. (a) Confocal fluorescence images of FITC-linked 6-arm-PEG-catechol grafted islets and (b) fluorescent intensity of dark area on the merge image.

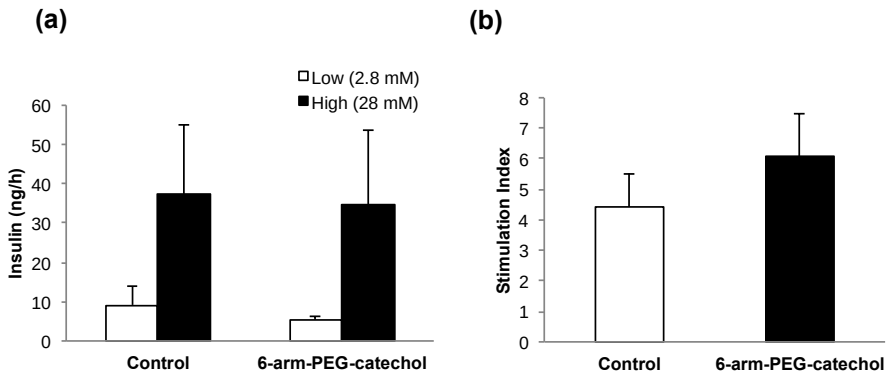

Figure 3.4. (a) The glucose stimulated-insulin secretion (GSIS) of unmodified and 6-arm-PEG-catechol grafted at low (white bar, 2.8 mM) or high (Black bar, 28 mM) glucose solution for 2 h (n=5) (b) Stimulation index (SI) of unmodified and 6-arm-PEG-catechol grafted islets (n=5). Data were expressed as mean of ± SEM.

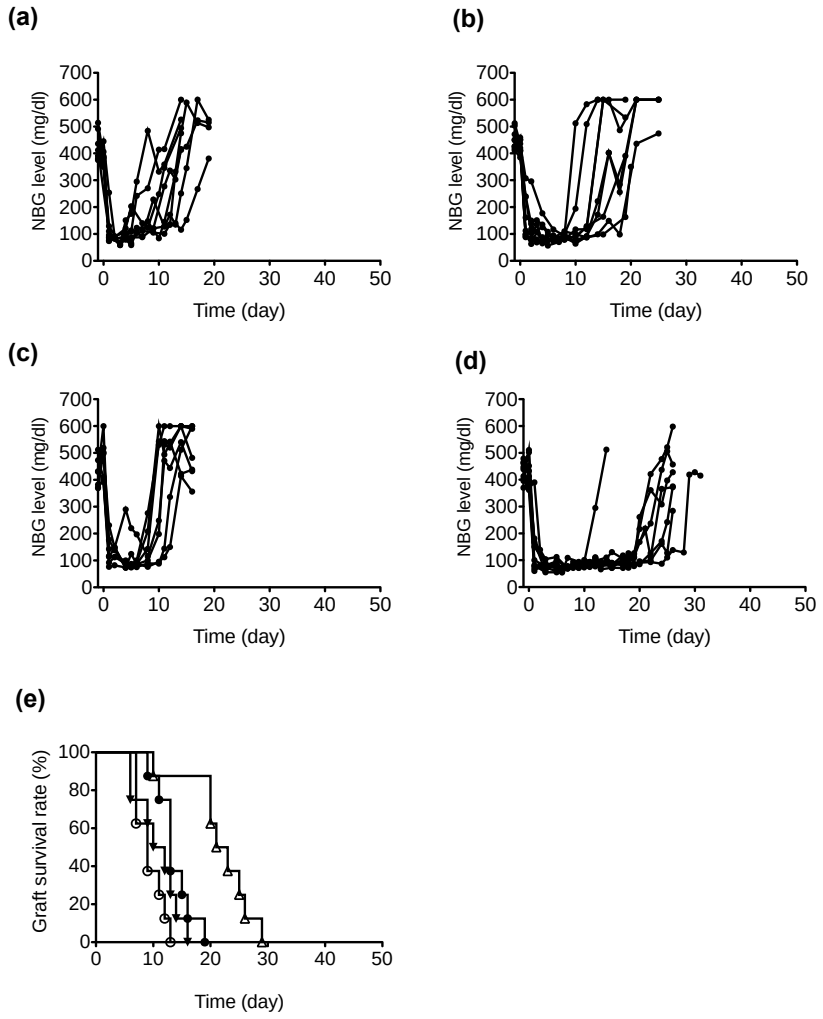

Figure 3.5. Nonfasting blood glucose level after islet transplantation into diabetic mice. (a) unmodified islet recipients (n=8), (b) 6-arm-PEG-catechol grafted islet recipients (n=9), (c) unmodified islet recipient with tacrolimus treatment (n=7), (d) 6-arm-PEG-catechol modified islet recipients with tacrolimus treatment (n=8), (e) Graft survival rate of each group. (▼) unmodified islets recipients, (●) 6-arm-PEG-catechol grafted islets recipients, (○) unmodified islets recipients with tacrolimus treatment and (Δ) 6-arm-PEG-catechol grafted islets recipients with tacrolimus treatment.

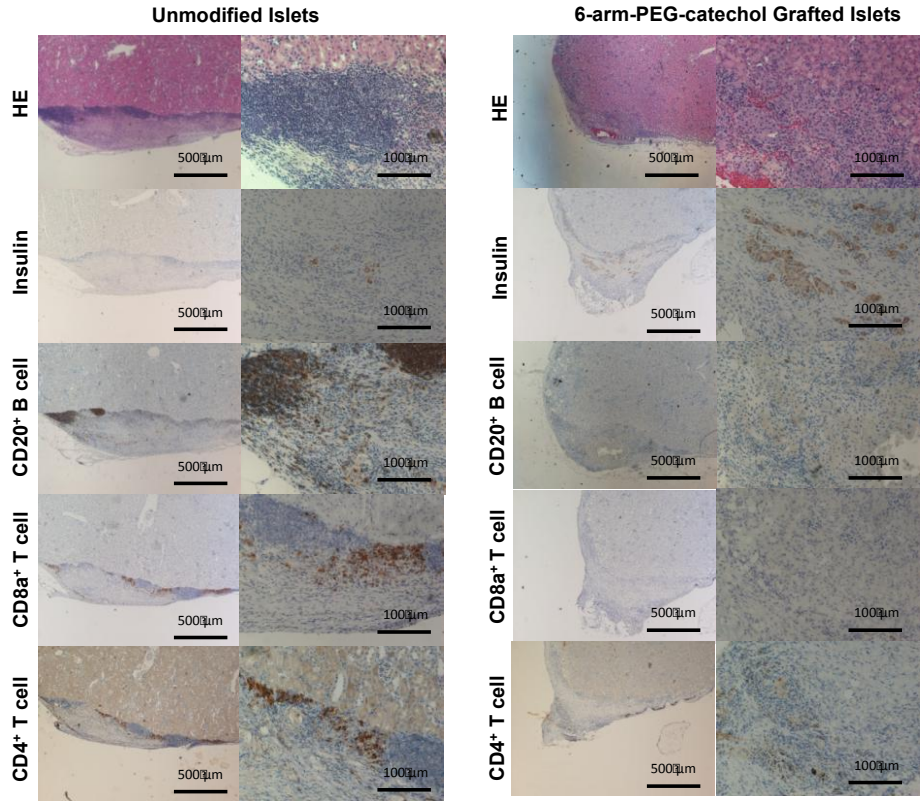

Figure 3.6. Immunohistochemical analysis of transplanted islets when treated with Tacrolimus. (HE; Hematoxylin and eosin stain)

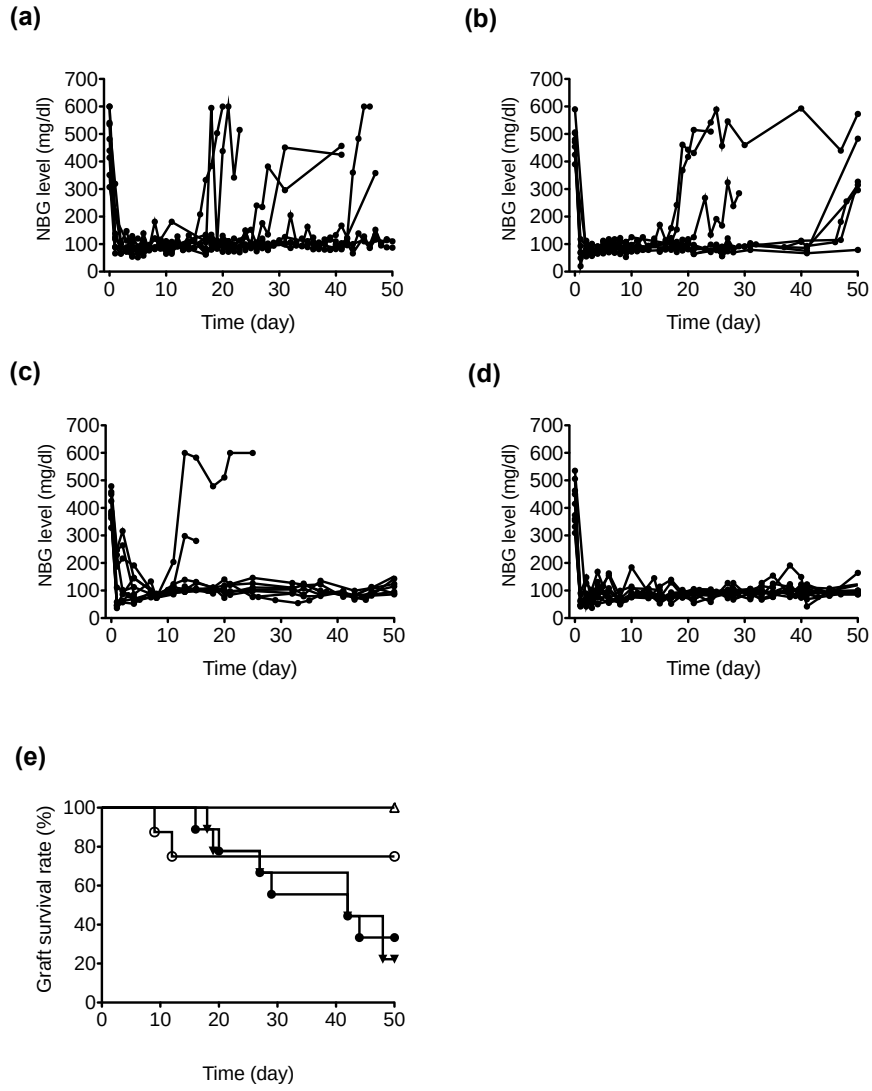

Figure 3.7. Nonfasting blood glucose level after islet transplantation into diabetic mice. (a) unmodified islet recipients with anti-CD154 mAb treatment (n=9), (b) 6-arm-PEG-catechol grafted islet recipients with anti-CD154 mAb treatment (n=8), (c) unmodified islet recipients with tacrolimus treatment and anti-CD154 mAb (n=9), (d) 6-arm-PEG-catechol modified islet recipients with tacrolimus and anti-CD154 mAb treatment (n=10), (e) Graft survival rate

of each group. (▼) 6-arm-PEG-catechol grafted islets recipients with anti-CD154 mAb treatment, (●) unmodified islets recipients with anti-CD154 mAb treatment, (○) unmodified islets recipients with both tacrolimus and anti-CD154 mAb treatment and (Δ) 6-arm-PEG-catechol grafted islets recipients with both tacrolimus and anti-CD154 mAb treatment.

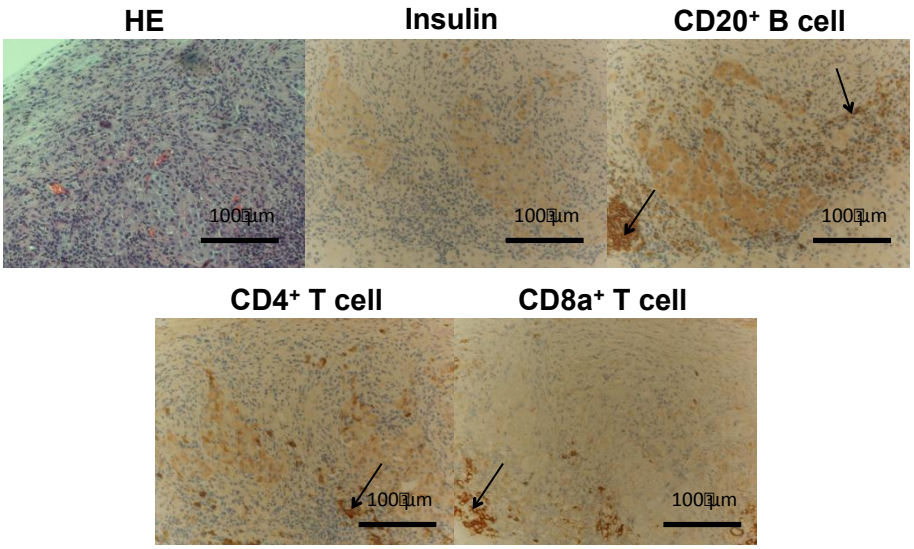

Figure 3.8. Immunohistochemical analysis of transplanted islets when treated with tacrolimus and anti-CD154 mAb. (HE; Hematoxylin and eosin stain)

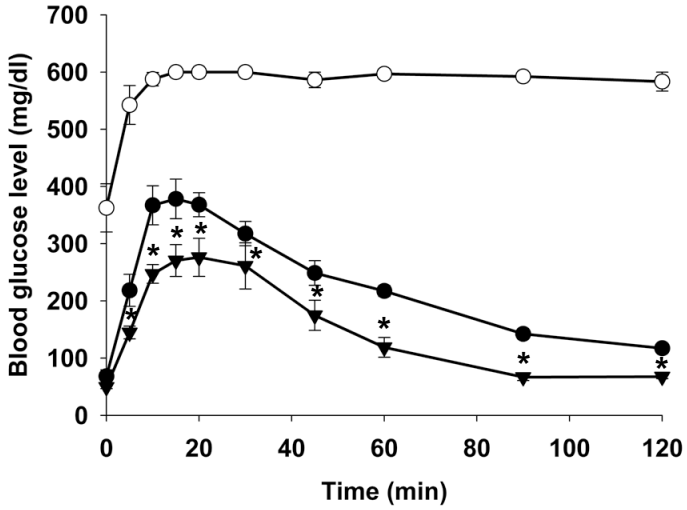

Figure 3.9. The intraperitoneal glucose tolerance test (IPGTT) of normal (●) (n=6), diabetic (○) (n=6), 6-arm-PEG-catechol grafted islets treated with Tacrolimus and anti-CD154 mAb (▼) 50 days after transplantation. Data were expressed as mean ± SEM (n=10), (*P<0.01 vs. diabetic group, t-test).

3.4. Discussion

In this study, we developed a new convergent protocol for the xenotransplantation of pancreatic islets by surface camouflage using polymer and immunosuppressive drugs such as tacrolimus and anti-CD154 mAb. Six-arm-PEG-catechol effectively covered the islet surfaces, and the catechol moiety was used to play a major role in conjugating 6-arm-PEG on the islet surface. The catechol moiety is a component extracted from *Mytilus edulis* foot protein 1 (Mefp-1), which is known as mussel adhesive proteins, and it works as a surface-independent anchor molecule [18, 19]. Oliogo or multimerized catechol molecules can bind to the versatile surfaces including hydrophobic, fluorine-containing materials and even extra hepatic areas [20-23]. Since the isolated pancreatic islets were fully covered by collagen matrix, 6-arm-PEG-catechol would be conjugated onto collagen matrix without their conjugation on the cell membrane under the optimized condition. If the conjugation time was increased, PEG molecules could diffuse into islets and reduce cell viability. In this study, 6-arm-PEG-catechol completely covered the surface of islets without causing any damage on islet cell viability and their functionality.

In xenotransplantation, when the survival time of 6-arm-PEG-catechol grafted islets and unmodified islets were compared, the survival time of islets was not improved by grafting of 6-arm-PEG-catechol. Although surface camouflage might inhibit the immune cell recognition and infiltration, it would not prevent the activation of immune cells and secretion of cytokines

from activated immune cells. To overcome the limitation of surface camouflage technology of islets, we introduced a low dose of tacrolimus as a calcineurin inhibitor, which was used in what is known as the “Edmonton protocol” [1, 24, 25]. When a low dose of tacrolimus was continuously administered after the transplantation of 6-arm-PEG-catechol grafted islets, its mean survival time was prolonged twice since tacrolimus inhibited the secretion of cytokines by interfering with the production of IL-2 by T-cells, thereby attenuating the recruitment of $CD20^+$ B cells and $CD8^+$ T cells around the transplanted site [26], $CD4^+$ T cells, however, were still present around the transplanted site but without infiltrating, and this activation of $CD4^+$ T cells was the reason that the survival time of the transplanted islet was not significantly increased.

Finally, anti-CD154 mAb (co-stimulatory receptor binding molecule) was additionally administered with tacrolimus to the recipients that 6-arm-PEG-catechol grafted islets were transplanted. All recipients in this group maintained their normal blood glucose level up to day 50 of transplantation. In addition, 6-arm-PEG-catechol grafted islets were well preserved around the transplanted site at day 50 of transplantation without recruiting $CD20^+$, $CD4^+$, and $CD8^+$ T cells around the 6-arm-PEG-catechol grafted islets.

Several clinical studies have reported that anti-CD154 mAb is a potent drug candidate for improving the islet graft survival rate. However, anti-CD154 mAb has not been approved for the current drug market because of its potential for triggering thromboembolism [27, 28]. In this study, only 20% of the established anti-CD154 mAb concentration (0.5 mg/mice) with a

low dose of tacrolimus was used in combination to prevent host immune reactions. Therefore, the synergistic effect of surface camouflage using 6-arm-PEG-catechol and anti-CD154 mAb and tacrolimus could reduce adverse effects of the immunosuppressive drugs by enhancing their immunoprotective effects.

3.5. Conclusion

The newly developed protocol of the surface camouflage of pancreatic islets using 6-arm-PEG-catechol and co-administration of immunosuppressive drugs such as anti-CD154 mAb and tacrolimus would certainly be an effective combination therapy for the pancreatic islet xenotransplantation. The effects of convergent technology would be summarized as shown in Fig. 3.10: i) Surface camouflage using 6-arm-PEG-catechol prevented the infiltration of immune cells and inhibited the antigen recognition; ii) The low dose of tacrolimus alleviated the inhibition of cytokine secretion from host immune cells. iii) anti-CD154 mAb prevented T cell activation.

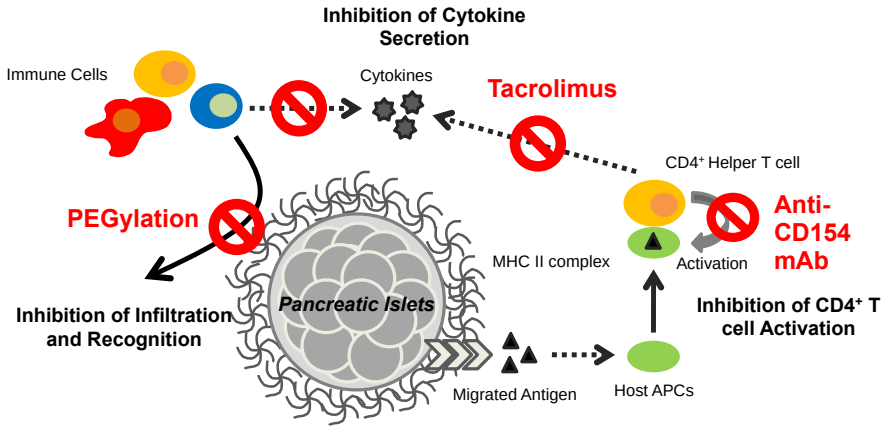

Figure 3.10. Illustration of convergent protocol for islet immunoprotection

3.6. References

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Chapter 4. Effects of Surface Camouflaged Islet Transplantation on Glucose Control and Reduction of Pathophysiological Progression in a *db/db* Type 2 Diabetic Mouse Model

4.1. Introduction

Type 2 diabetes is characterized by a decrease in insulin secretion and an increase in insulin resistance [1-5]. Most obese type 2 diabetes show an insulin resistance associated with hyperinsulinemia in the early stage of the disease [6]. As the disease progresses, the functions of pancreatic beta cells are gradually deteriorated, finally developing severe insulin deficiency [7]. The morphological damage of the pancreatic islets in obese type 2 diabetes is induced by compensatory overexpression of insulin and the resultant exhaustion of beta cells in response to the increased insulin demand. In addition, a high prevalence of non-alcoholic fatty liver has been associated with damage observed in the liver in a *db/db* type 2 diabetic mouse model [8]. Insulin resistance has been shown to be one of the causes for these non-alcoholic fatty liver diseases (NAFLD) [9, 10]. Accordingly, several clinical studies have used insulin sensitizers such as metformin in insulin-resistant NAFLD patients [11].

In these respects, we expected that additional exogenous insulin administration could compensate for excessive demand for insulin secretion from the beta cells, and furthermore, deter the progression of liver damage and NAFLD progression in type 2 diabetes. The insulin therapy in early stage of obese type 2 diabetes had been found to improve both insulin action and secretion, thereby overcoming insulin resistance [12]. Thus, basal-bolus insulin therapy is one of the choices for regulating the blood glucose level of type 2 diabetic patients, offering a way to closely stimulate natural insulin

delivery in the clinic [13, 14]. Malmbeg *et al.* demonstrated that the unfavorable long-term prognosis for myocardial infarction could be improved by insulin treatment for diabetic patients [15]. In addition, Schwartz *et al.* also showed that a regimen of premixed insulin in combination with metformin was as effective as but much less expensive than a triple hypoglycemic drug therapy [16]. Their insulin therapy significantly reduced the glycosylated hemoglobin (HbA1C) level when the HbA1C baseline was above 9.5% [17]. However, the insulin therapy has shown many concerns with respect to hypoglycemia, patient willingness, and incompliance. Therefore, islet transplantation has been shown to be another alternative by which to properly deliver insulin to diabetic patients.

Several studies have used islet transplantation for the treatment of the type 2 diabetes in a mouse model [18-24]. Gates *et al.* have ameliorated the abnormalities of obese-hypoglycaemic by allogeneic implantation of islets [18, 19]. They used a Millipore bag as a device for preventing graft rejection, but it had limitations in sensing glucose concentration and in maintaining islet viability [25]. Furthermore, it was reported that although MIN-6 cell transplantation decreased hyperglycemia in *db/db* mice over 100 days after transplantation, the transplanted MIN-6 cells had become the cancerous tissue [26]. Andersson *et al.* showed that intrasplenic islet transplantation was failed to cure obese-hyperglycemic mice [21]. Barker *et al.* claimed that islet transplantation was not suitable as a treatment for *db/db* mice [23]. Katsuragi *et al.* examined that transplanted islet through the portal vein prevented the diabetic progression in Otsuka Long Evans Tokushima Fatty (OLEFT) rats.

They reported on the possibility of transplanted islets to prevent the pancreas and mesangial matrix in the renal glomeruli from undergoing morphological changes [27]. Collectively, there were many studies reporting about the advantage of insulin therapy for deterring pathophysiological progression of type 2 diabetes. However, it was hard to effectively deliver the insulin secreted from transplanted islets to type 2 diabetic patients because the grafted islets were easily rejected by host immune reaction. If transplanted islets would be survived for long-term periods without the immune rejection, transplanted islets would effectively deter the progression of type 2 diabetes. To ameliorate these limitations in the treatment regime and to minimize the immune reaction after transplantation, and based on the previous studies, we introduced an islet surface modification technology combined with immunosuppressive medication as a treatment for type 2 diabetes [28, 29]. In the previous study, the surface camouflage of pancreatic islet using 6-arm-PEG-catechol and the administration of tacrolimus (FK506) and anti-CD154 mAb (MR-1) were effective in preventing immune reactions against transplanted islets [28].

In this study, 6-arm-PEG-catechol grafted islets were transplanted into *db/db* mice with co-administration of anti-CD154 mAb and tacrolimus to investigate the curing effect of type 2 diabetes. Furthermore, inhibition effects of this protocol on pathophysiological damage progression in type 2 diabetes were evaluated in a *db/db* type 2 diabetic mice model.

4.2. Materials and Methods

4.2.1. Immobilization of 6-arm-PEG-catechol on the surface of pancreatic islets

Pancreatic islets were isolated from Sprague-Dawley (SD) rats (male, 8-weeks old, Orient Bio Inc., Seongnam, South Korea). SD rats were anesthetized by pentobarbital, followed by abdominal laparotomy. Ca^{2+} and Mg^{2+} free Hank's balanced salt solution (HBSS, Sigma) containing 12 ml collagenase type IV (1.5 mg/ml, Sigma) was injected via common bile duct to the pancreas. The expanded pancreas was extracted and incubated for 11 min at 37℃ for the digestion of extracellular structures. After mechanical disruption of expanded pancreas by shaking, islet cells were separated by discontinuous Ficoll™ PM400 (Amersham Biosciences AB, Uppsala, Sweden) density gradient. Isolated islets were then handpicked and cultured in RPMI-1640 (Sigma) medium with 10% fetal bovine serum (FBS) at 37℃ under the humidified atmosphere containing 5% CO_2. The 6-arm-PEG-catechol molecules were chemically immobilized onto the surfaces of the isolated islets as mentioned in the literature [28]. After 2 days culture, the isolated islets were washed twice with the HBSS (pH 8.0), and HBSS containing 0.25% (w/v) 6-arm-PEG-catechol were added to the islets to chemically immobilize 6-arm-PEG-catechol onto the islet surfaces. The islets were then incubated for 1 h at 37℃ in RPMI-1640 media containing 10% FBS, followed by washing 3 times with fresh HBSS. All of the animal experiments were carried out according to the guidelines of the Institute of

Laboratory Animal Resources, Seoul National University (IACUC no. SNU-070822-5)

4.2.2. Transplantation of 6-arm-PEG-catechol grafted islets

Six-week-old male *db/db* C57BL/KsJ mice (*db/db* group) and their lean non-diabetic heterozygous littermates, *db/m* mice (*db/m* group), were purchased from Japan SLC Inc. (Hamatsu, Japan). Spontaneously induced type 2 diabetic *db/db* mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylzine (16 mg/kg) after being housed for 2 weeks. The left kidney was exposed through a lumbar incision and capsulotomy was carried out at the cadual layer of the left kidney, followed by the transplantation of 500 IEQ 6-arm-PEG-catechol grafted islets (IT group). The same volume of medium without islets was injected into the kidney capsules of *db/db* mice as sham-operated mice (Sham group). Thus, there were four following groups: *db/db*, *db/m*, IT group and Sham group. Also, anti-CD154 mAb (0.2 mg/mouse) was injected at 0, 2, 4, and 6 day of post-transplantation and tacrolimus (0.2 mg/kg) was daily injected into the recipient intrapertoneally.

In addition, intraperitoneal glucose tolerance test (IPGTT) was performed to measure the glucose responsiveness at day 5 after islet transplantation. Each group was fasted for 6 h before the injection of 20% glucose (10 μl/g) solution intrapertoneally. Then, the blood glucose level was measured from the tail vein using portable glucometer (Super glucocard II, Arkray, Kyoto, Japan) at 0, 15, 30, 60, and 90 min after glucose injection.

4.2.3. Immunohistochemistry

Six-arm-PEG-catechol grafted islets were transplanted in the kidney capsule of *db/db* mice. At day 14 of islet transplantation, the kidney was retrieved and fixed in neutral 4% paraformaldehyde-phosphate-buffered saline, and embedded in paraffin. The kidney sections were deparaffinized in xylene and then rehydrated in serially graded alcohol. The slides were pre-incubated in dextran solution and 4% bovine serum albumin for 30 min to reduce non-specific binding after washing with distilled water. The slides were stained with hematoxylin and eosin (H&E), anti-insulin (Abcam, Cambridge, MA), anti-$CD4^+$ (Abcam Inc., Cambridge, MA), anti-$CD8^+$ (BioLegend, San Diego, CA), and anti-$CD20^+$ (Santa Cruz Biotechnology Inc., Santa Cruz, CA) antibody, respectively. At day 3 and 14 of islets transplantation, the pancreas, kidney, liver, and abdominal adipose tissue were stained with H&E and their morphological changes were evaluated. The sizes of adipocyte and pancreatic islet were also measured using the ACT-2U imaging software (Nikon, Tokyo, Japan). Three slides of each group were analyzed.

4.2.4. Measurement of body weight and analysis of blood samples

The body weight and food uptake were measured every 2 or 3 days, and non-fasting blood glucose (NBG) levels were measured by drawing blood from the tail veins using a portable glucometer (Super glucocard II, Arkray, Kyoto, Japan). Insulin concentrations in serum were determined using the ELISA (rat/mouse insulin ELISA kit, Millipore, Billerica, MA). In addition,

liver toxicity (Aspartate aminotransferase; AST, alanine aminotransferase; ALT) was measured using FUJI DRI-CHEM 3500 (FUJIFILM, Tokyo, Japan).

4.2.5. Statistical analysis

All the data were expressed as mean ± SEM. Statistically analysis was carried out using unpaired *t*-test or ANOVA one-way test. A *P* value of less than 0.05 was considered to be statistically significant.

4.3. Results

4.3.1. Pathophysiological analysis after 6-arm-PEG-catechol islet transplantation

Photomicrographs of the pancreatic islets in *db/db*, *db/m*, and IT groups at day 14 of their transplantation were illustrated in Fig. 4.1. The pancreatic islets of *db/db* group appeared much larger than those of *db/m* group and became multi-nodular due to the intra-insular proliferation of fibrous connective tissue. On the other hand, the pancreatic islets of IT group appeared as the normal although the intensity of insulin immunostaining was less than that of *db/m* group. The area quantification of insulin positive cells in *db/db* group was 17920 ± 7372 μm^2. Contrastively, the area of IT group was 6379 ± 1661 μm^2, which was 2.8-fold smaller than that of *db/db* group (Fig. 4.1b). This result indicated that islet transplantation could inhibit the enlargement of pancreatic islets and proliferation of fibrous connective tissue, which are usually induced in type 2 diabetic mice in order to compensate for

hypoglycemia. Thus, the islet transplantation further delayed the destruction of pancreatic islets by reducing the size expansion of islet in *db/db* mice.

The color of the liver surface in *db/db* group was white-yellowish as a result of accumulating fat. On the other hand, the color of the liver surface in IT group was red as the normal, which was similar with those of *db/m* group (Fig. 4.2a). The hepatocytes in *db/db* group contained empty vacuoles in hematoxylin-eosin-stained sections, as shown in Fig. 4.2a. The fat was infiltrated into vacuole within the cytosol of hepatocytes in *db/db* group; however, the fat infiltration into the liver was not observed in IT group. To confirm the effect of 6-arm-PEG-catechol grafted islet transplantation on alleviating the liver toxicity in *db/db* mice, both ALT and AST levels in the serum of *db/db*, *db/m* and IT group were analyzed, respectively, using FUJI DRI-CHEM 3500 (FUJIFILM, Tokyo, Japan) at day 14 after 6-arm-PEG-catechol grafted islet transplantation (Fig. 4.2b, c). ALT and AST levels in *db/db* group were 302 ± 45 U/l and 262 ± 12 U/l, respectively. On the contrary, ALT and AST levels in IT group were statistically decreased to 151 ± 22 U/l and 176 ± 22 U/l, respectively. These results demonstrated that 6-arm-PEG-catechol grafted islet transplantation in a *db/db* diabetic mouse model partly recovered the liver from high fat accumulation and damage.

Adipocytes play an important role in regulating energy expenditure, food intake, and glucose metabolism. Therefore, the adipocyte is an important indicator for metabolic alterations related to the insulin resistance [26]. As shown in Fig. 4.3, the size of an adipocyte, which was measured at the same

time point of IT group in *db/m* and *db/db* groups, were 1082 ± 116, and 3259 ± 376.4 μm^2, respectively. On the other hand, the adipocyte size of IT group was 1675 ± 221 μm^2, which was statistically decreased compared to that of *db/db* group. These findings indicated that transplanted 6-arm-PEG-catechol grafted islets supplied a sufficient amount of insulin in place of host beta cells so that there was no need to compensate for hyperglycemia by enlarging the size of islet and the size of adipocytes.

4.3.2. Blood glucose control and IPGTT

The NBG levels of the sham group and *db/db* group were consistently increased with the progression of type 2 diabetes. However, the NBG level of *db/db* mice, which received 6-arm-PEG-catechol grafted islets, maintained normoglycemia for one week after transplantation with the co-administration of anti-CD154 mAb and Tacrolimus (Fig. 4.4a). In addition, no significant changes in body weights were observed in either of the *db/db* group, sham group, or IT group (Fig. 4.4b). To clarify the causes of change in blood glucose level, daily food uptakes in each group were measured. The amount of food uptake was significantly decreased in both sham group and IT group, compared to that in *db/db* group for the first 3-4 days of post-transplantation (Fig. 4.4c). These results showed that the cause of decreased food uptake was due to the surgical performances; however, the decreased food uptake due to surgical procedure was not the main cause of the NBG decrease. Even though the food uptakes in both sham and IT groups were decreased after the surgical

procedure, only NBG of IT group reached the plasma insulin level above 200 mg/dl. The blood glucose levels of sham group were remained high. This result indicated that the secreted insulin from the transplanted islets was responsible for decreasing the blood glucose level in the recipients.

The blood glucose level of IT group was started to increase at day 7 and returned to 400 mg/dl after 14 days of transplantation. This finding has shown that the transplanted islets did not maintain the normal blood glucose level of recipients over 1 week. To evaluate the reason of this phenomenon, the serum insulin level was analyzed. The insulin level of IT group was significantly increased at day 3 of transplantation; that is, the serum insulin level of IT group at day 3 (8.41 ± 2.08 ng/ml) was increased by 3.3-fold, compared to that in *db/db* group (2.56 ± 0.31 ng/ml). However, the serum insulin level measured at day 14 in IT group decreased to 4.23 ± 0.73 ng/ml (Fig 4.5). Collectively, this result indicated that the transplanted 6-arm-PEG-catechol grafted islets started to lose their function at day 7 and a beta cell function for regulating blood glucose level was significantly lost at day 14 after transplantation.

To confirm glucose responsiveness of transplanted 6-arm-PEG-catechol grafted islets, IPGTTs were carried out at day 5 of transplantation (Fig. 4.6). The blood glucose level of *db/db* group rapidly increased to 600 mg/dl within 20 min. On the other hand, the blood glucose level of IT group reached the highest concentration (~ 400 mg/dl) after 30 min of glucose injection and then gradually decreased afterwards, showing that the insulin

secreted from transplanted 6-arm-PEG-catechol grafted islet prevented a hasty increase of blood glucose level upon administration of high exogenous glucose solution.

4.3.3. Immunohistochemistry

To verify insulin secretion and immune cell recruitment around the transplanted 6-arm-PEG-catechol grafted islets at day 3 and 14 of post-transplantation, the left kidney containing transplanted islets was harvested. At day 3, insulin positive cells were detected around the transplantation site; however, CD4, CD8a and CD20 positive cells were rarely detected around the islet transplanted site. This result indicated that transplanted 6-arm-PEG-catechol grafted islets secreted sufficient amounts of insulin; in other words, islet functions were preserved even after the islet transplantation. At day 14 of transplantation, the mass of transplanted 6-arm-PEG-catechol grafted islet was preserved in the transplantation site; however, the intensity of insulin staining was lower than that of the transplanted 6-arm-PEG-catechol grafted islets at day 3. Even though the function of beta cells deteriorated over time, there was no evidence of severe immune cell recruitment around the transplantation site at day 14. Therefore, the combination protocol of surface camouflaged islets using 6-arm-PEG-catechol and the administration of immunosuppressive drugs was successful in inhibiting graft rejection after transplantation (Fig. 4.7).

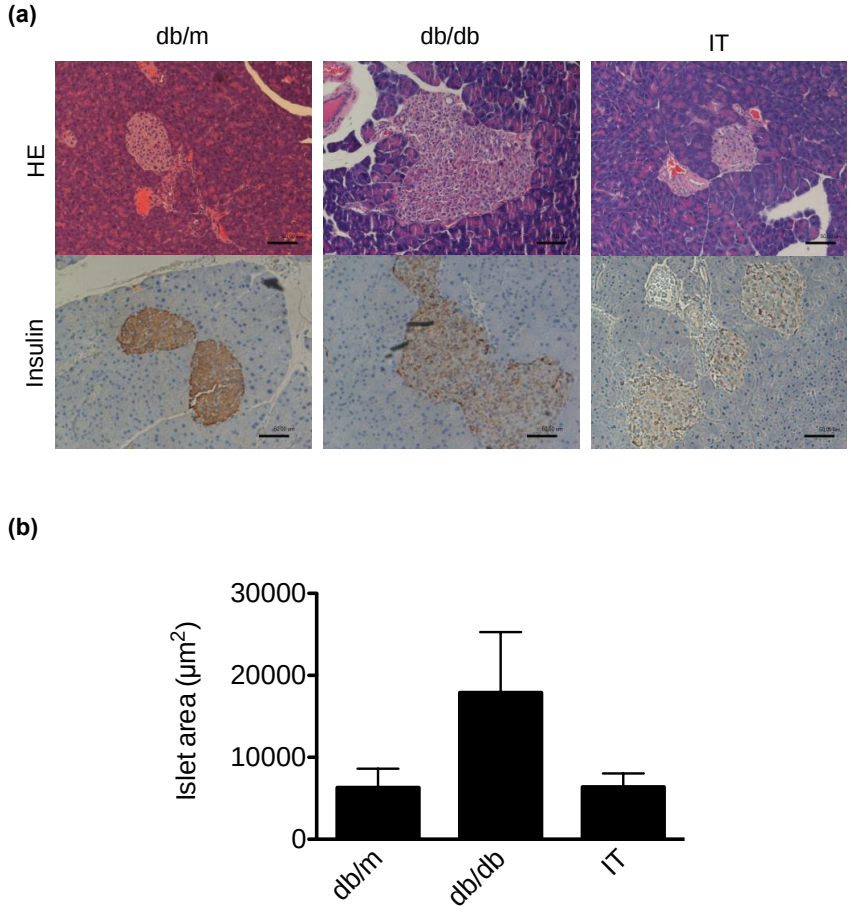

Figure 4.1. Morphology and size changes of islets in the pancreas at day 14 of 6-arm-PEG-catechol grafted islet transplantation in *db/m*, *db/db* and IT groups (a) Hematoxylin-eosin and insulin staining of pancreatic islets, Scale bar = 50 μm (b) the area of insulin-positive cells in the pancreas. Data were expressed as mean ± SEM

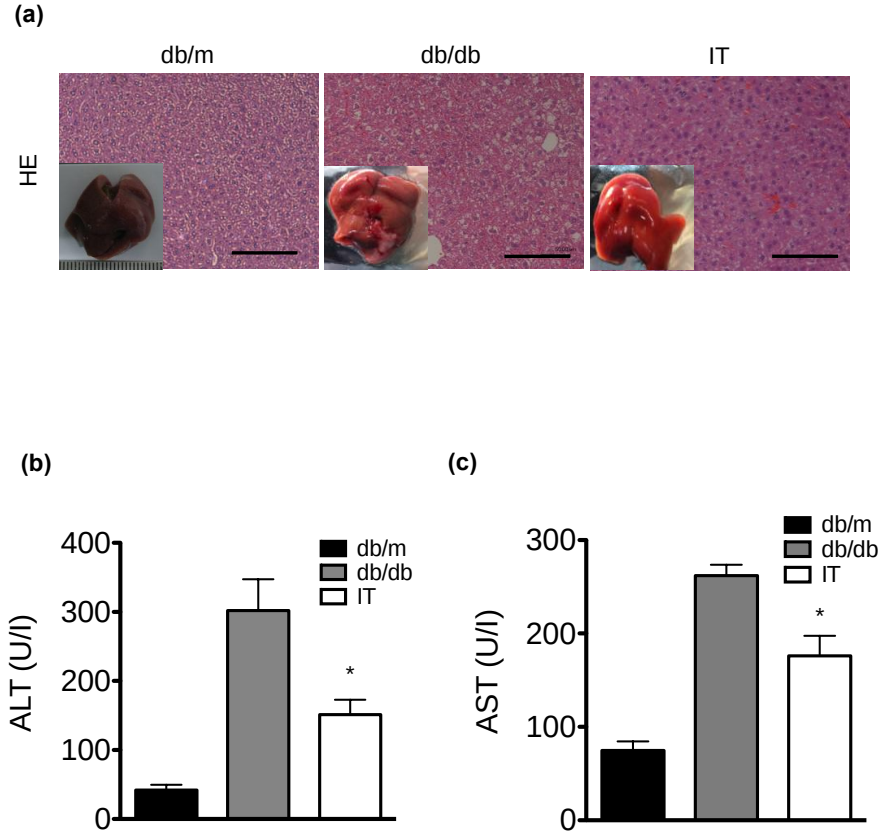

Figure 4.2. (a) The gross appearance and histological findings of the liver in the *db/m*, *db/db*, and IT groups at day 14 of 6-arm-PEG-catechol grafted islet transplantation. Scale bar = 100 μm (b) Alanine *amino*transferase (ALT) and aspartate aminotransferase (AST) level in the serum of *db/m*, *db/db*, and IT groups. Data were expressed as mean ± SEM (*db/m*; n=3, *db/db*; n=3, IT; n=5) (HE; Hematoxylin and eosin stain)

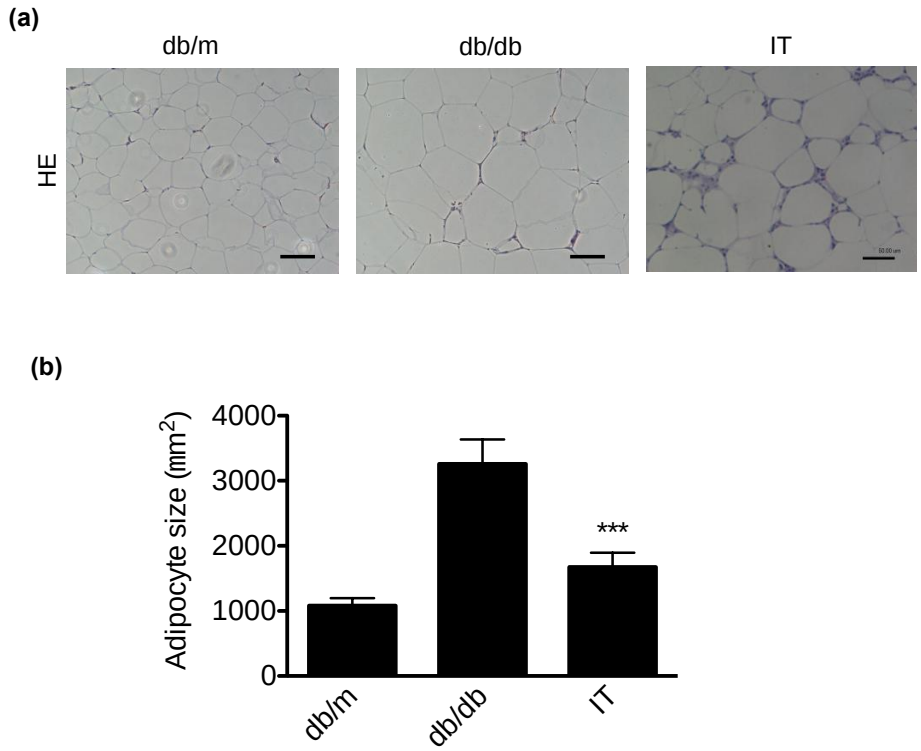

Figure 4.3. Adipocyte size and morphology changes at day 14 of 6-arm-PEG-catechol grafted islet transplantation (a) Histology of abdominal adipose tissue in *db/m*, *db/db*, and IT groups. Scale bar = 50 μm (b) Size of adipocyte in the *db/m*, *db/db*, and IT groups. Data were expressed as mean ± SEM (*db/m*; n=3, *db/db*; n=3, IT; n=5; for each slide, 21 adipocytes were measured at random with an image analysis system.); (***$P < 0.001$ vs. *db/db* group) (HE; Hematoxylin and eosin stain)

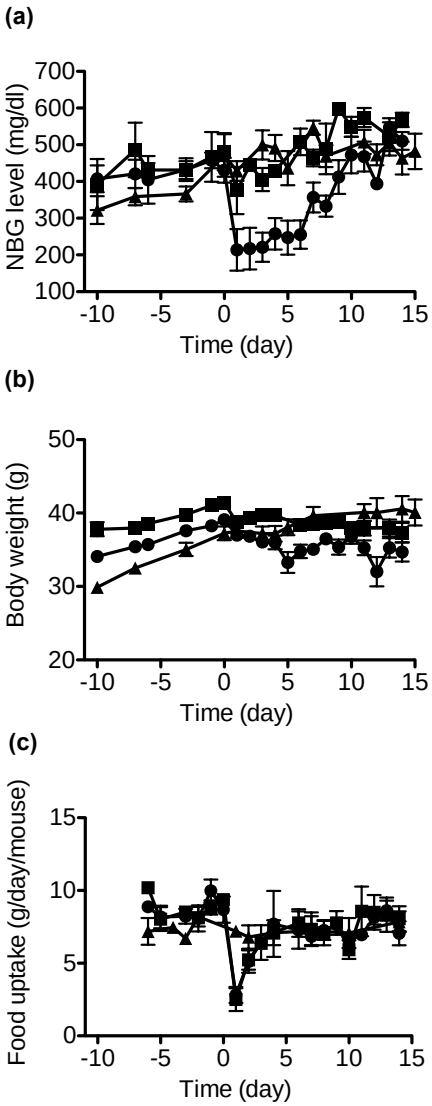

Figure 4.4. Time course of changes in (a) blood glucose, (b) body weight and (c) food uptake in *db/db* (black triangle), sham (black square) and IT (black circle) groups. Data were expressed as mean ± SEM (*db/db*; n=6, sham; n=3, IT; n=8)

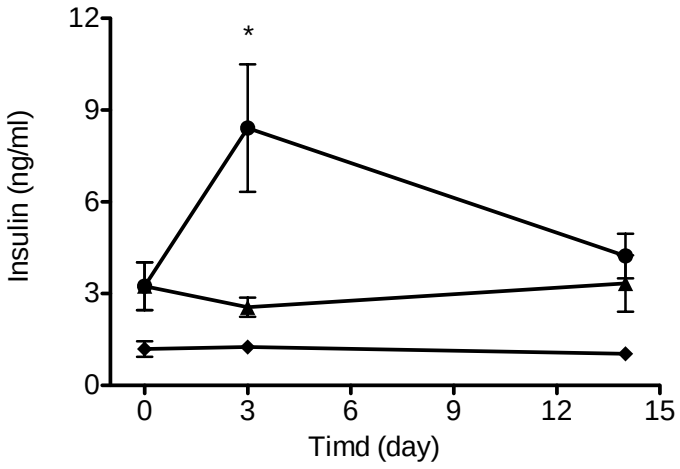

Figure 4.5. Fasting insulin serum levels at day 0, 3 and 14 after 6-arm-PEG-catechol grafted islet transplantation, Data were expressed as mean ± SEM (n=3), *db/m* (black diamond), *db/db* (black triangle) and IT (black circle) groups.

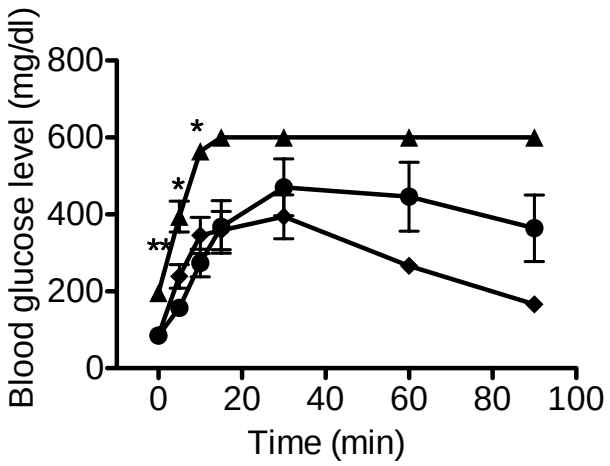

Figure 4.6. The intraperitoneal glucose tolerance test (IPGTT) of *db/m* (black diamond), *db/db* (black triangle), and IT (black circle) groups at day 5 of transplantation. Data were expressed as mean ± SEM (n= 4), (**P* < 0.05, ***P* < 0.01 vs. IT group)

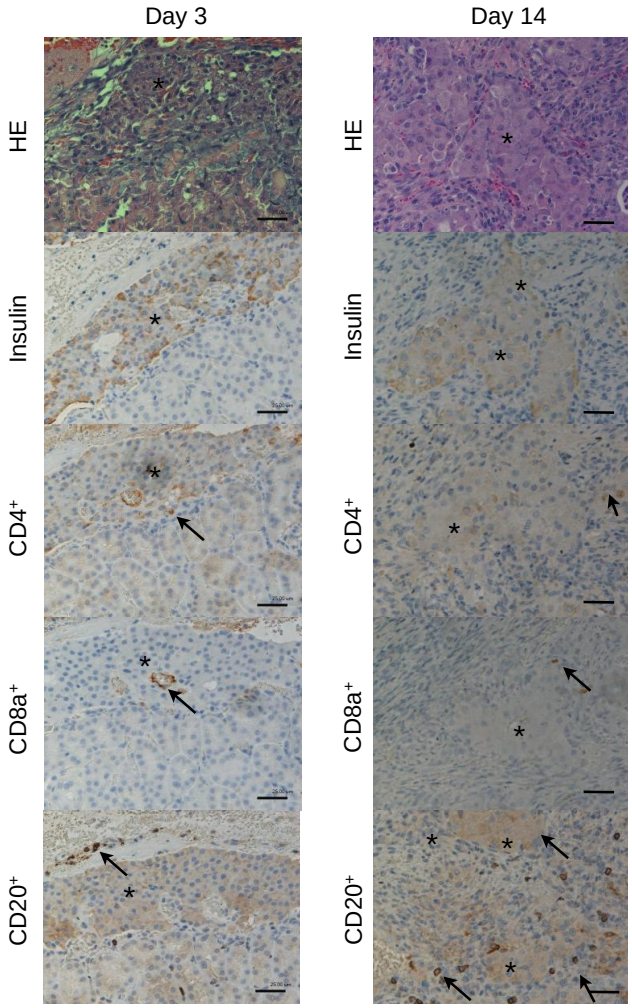

Figure 4.7. Immunohistochemical analysis of transplanted 6-arm-PEG-catechol grafted islets after 3 and 14 days of transplantation. Asterisk: transplanted islets, Scale bar = 25 μm. (HE; Hematoxylin and eosin stain)

4.4. Discussion

In this study, we investigated the therapeutic effects of surface camouflaged islets transplantation on the type 2 diabetes and its related pathophysiological phenomena in a *db/db* mouse model. When morphological expansion and exhaustion of islets were observed in the type 2 diabetic mice, and the expansion of the islet size was prominently due to their need to compensate for hyperglycemia and the resistance against insulin. However, the transplantation of 6-arm-PEG-catechol grafted islets prevented the size expansion of islet in the pancreas. The size of adipocytes is an indirect factor indicating the insulin resistance and glucose tolerance in the type 2 diabetes. Based on the severity of obesity in *db/db* mice, the size of adipocyte was also increased; however, the expansion of adipocyte was also significantly inhibited following the 6-arm-PEG-catechol grafted islet transplantation. At day 14, the size of adipocyte in IT group was much smaller than that of *db/db* group. At this point, insulin delivered from the transplanted 6-arm-PEG-catechol grafted islets might have affected the diminution of insulin resistance and enhanced lipolysis in *db/db* mice. Thus, the enlargement of adipocytes in the 6-arm-PEG-catechol grafted islet transplanted *db/db* mice were effectively inhibited.

Bugianesi *et al*. reported that NFALD was related to insulin resistance, obese, and hypertension. The AST and ALT values are sensitive indicators of hepatocellular injury, and these values are associated with insulin resistance, obesity and type 2 diabetes [30, 31]. The AST and ALT values are also

strongly associated with fat accumulation in the liver and diminution of these values indicates that the liver had somewhat recovered from its injured condition. The degree of fat accumulation, ALT and AST values of IT group were significantly decreased, compared to those of *db/db* group, indicating that the transplanted islets could reduce the degree of liver injury. Insulin secreted from transplanted 6-arm-PEG-catechol grafted islets effectively delivered glucose inside the cell, thereby decreasing the insulin resistance in the liver. Therefore, the excessive accumulation of fat in the liver was effectively diminished. Also inflammation was observed to be significantly decreased in the liver of IT group, but a strong in *db/db* group.

In the previous study, we evaluated that islet surface modification was effective in inhibiting the immune cell activation and infiltration *in vitro* and *in vivo* [32, 33]. PEG conjugation to islet did not elicit any activation of co-cultured immune cells such as lymphocytes, macrophages and splenocytes. In addition, the synergistic effect of islet PEGylation and administration of immunosuppressive drug into the diabetic recipient was observed as well [28]. PEG immobilization prevented the infiltration of immune cells into the transplanted islets and immunosuppressive drugs alleviated the immune cell and cytokine activities [32, 34]. Thus, the PEGylation onto the surface of islets helped provide effective therapy over one year when accompanied with immunosuppressive drugs. In addition, a few rare T and B cells were observed around the PEGylated islets after the first 1 year of post-transplantation [35]. The same immunoprotection protocol optimized for prolonging the graft survival time was applied to type 2 diabetic mice to evaluate its effects in

inhibiting the pathophysiological disease progression. When 6-arm-PEG-catechol grafted islets were transplanted and both anti-CD154 mAb and tacrolimus were administered to the mice, the blood glucose level of islet recipients was effectively decreased down to 200 mg/dl, and the normal blood glucose level was maintained for one week. The blood glucose level started to gradually increase from day 7 and eventually reached hyperglycemia at day 14 of islet transplantation. When the serum insulin level was high, a normal blood glucose level was observed, indicating that the insulin secreted from the transplanted islets was responsible for the regulation of blood glucose level. In addition, transplanted islets had insulin responsiveness to glucose and their clearance ability was somewhat increase in IT group. Following the injection of high exogenous glucose solution, a rapid increase of blood glucose level was prevented in the recipients transplanted with islets. On the other hand, a greater amount of insulin was necessary for *db/db* group to regulate hyperglycemia because of insulin resistance. The amount of secreted insulin from *db/db* mice was not enough to regulate the blood glucose level to maintain normal glycaemia. Moreover, the insulin staining of transplanted 6-arm-PEG-catechol grafted islet after 14 days of transplantation was very faint. These results have shown that transplanted 6-arm-PEG-catechol grafted islets were also exhausted at day 14 of transplantation.

The ultimate goal of this study was to demonstrate the feasibility of islet camouflage technique used in combination with immunosuppressive drugs for the treatment of type 2 diabetes. Even though the graft survival time did not increase dramatically, we have verified that surface camouflage islets

using 6-arm-PEG-catechol effectively controlled blood glucose level for at least 2 weeks and also inhibited pathophysiological deterioration of type 2 diabetes during that time by way of reducing islet size expansion in the pancreas, preventing adipocyte size expansion and fat accumulation in the liver (Fig. 4.8). Therefore, the present islet transplantation would help to successful inhibit progression of type 2 diabetes in various therapeutic approaches, thwarting complications related type 2 diabetes progression. This proposed study is still under improvement. To prolong the survival of grafted islets in type 2 diabetes based on additional therapeutic approaches, genetic engineering or protein delivery would be used to improve insulin sensitivity and reduce insulin resistance. Although this proposed study would not be the first choice in the treatment, the research protocol can be used as a complementary therapy to oral hypoglycemic drugs for type 2 diabetes mellitus.

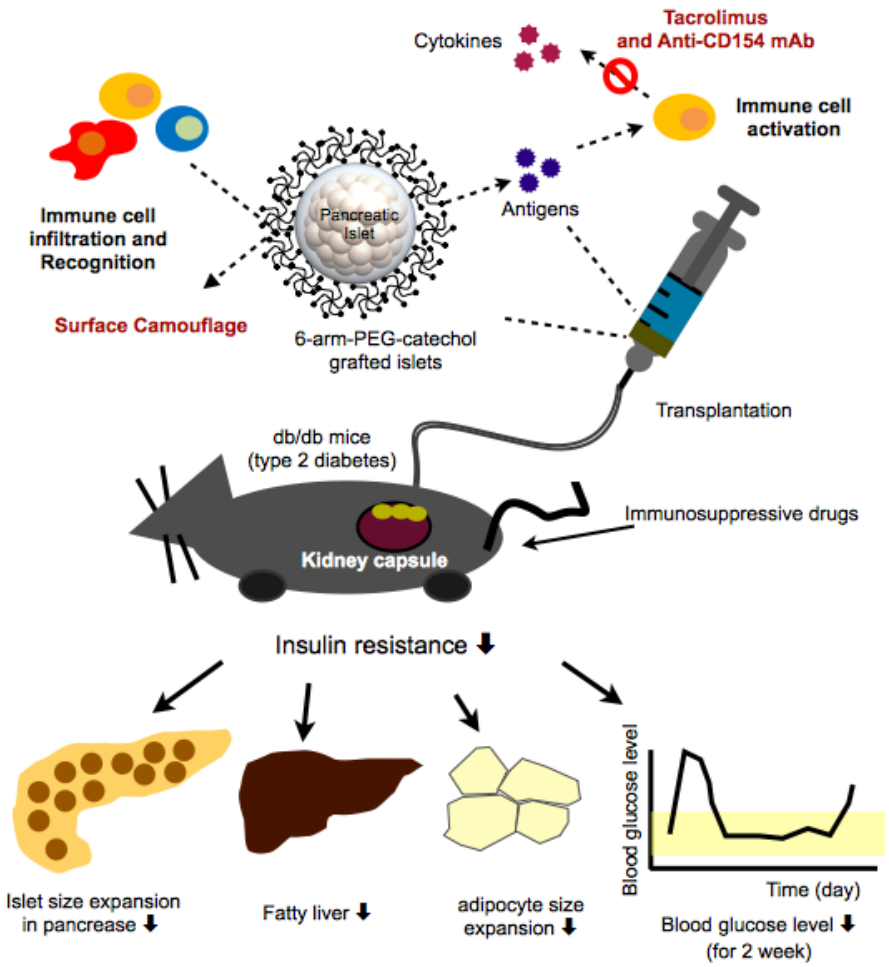

Figure 4.8. Illustration of surface camouflaged islets transplantation for treatment of type 2 diabetes

4.5. References

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Chapter 5. Immunoprotective Effect of PEGylation and Immunosuppressive Agents on Repeated Allotransplantation of Pancreatic Islets in Sensitized Diabetic Rat

5.1. Introduction

Despite continuous administration of immunosuppressants such as daclizumab, sirolimus and tacrolimus to prevent host immune reactions during transplantation, islet graft failure may still ensue and transplantation must be repeated [1]. The islets of the second transplantation, however, when compared to with those of the first-time islet transplantation to non-sensitized recipients, might be more rapidly rejected due to sensitized immune reactions of the humoral and cellular immunities induced by the first transplanted islets.

Surface camouflage of pancreatic islets using biocompatible polymers has been proposed as an alternative approach to protect transplanted islets against immune reactions [2-9]. The surface-immobilized polymers could then prevent host immune cells from infiltrating into the transplanted islets, thereby inhibiting humoral and cellular immunities. In the previous studies, we have demonstrated that the surface modification of islets using poly(ethylene glycol) (PEG) could protect allotransplanted islets against immune reactions. In particular, when a low dose of cyclosporine A (CsA) was daily administered, PEG grafted (PEGylated) islets would synergistically maintain their viability for more than one year in rats without any adverse effects [10-13].

However, repeated islet transplantation resulted in an accelerated rejection of the graft because the sensitized immune system had been sensitized with the previously rejected islets [14-17]. This accelerated graft rejection is species specific, and the antigens involved in the accelerated

rejections are shared by different strains within the same species [16]. Therefore, it is necessary to develop a new technology for protecting the islets of repeated transplantations against aggressive sensitized immune reactions. Co-stimulatory blockade (anti-CD154 mAb) has been reported to fail in a long-term survival of retransplanted islets and to prevent the activation of sensitized anti-donor antibodies [15, 18]. The α1-antitrypsin, a serum protease inhibitor possessing anti-inflammatory property, could induce immune tolerance during islet allograft transplantation in the same-strain mice. However, retransplantation of islets would not be accepted into third-strain mice [17]. These data indicate that there are still barriers to overcome for successful islet transplantations.

In this study, we determined whether a new PEGylation remedy would be effective in recipients that had been sensitized by a repeated islet allotransplantation. To this end, we evaluated the combination effect of CsA and anti-CD4 mAb (OX-38) on the viability and functionality of PEGylated islets in the second allotransplantation in diabetic rats.

5.2. Materials and Methods

5.2.1. Animals

Inbred F344 male rats, 7-8 weeks of age, and outbred Sprague-Dawley (SD) male rats, 8 weeks of age, were used as recipients and donors, respectively. Animals were purchased from Orient Bio Inc. (Gyeonggi, South Korea) and were housed in ventilated cages under a specific pathogen-free

condition. All animal procedures were approved by the Institutional Animal Care and Use Committee, which had been certified by the Institute of Laboratory Animal Resources of Seoul National University.

5.2.2. PEGylation onto the islet surface

Pancreatic islets were isolated from the outbred male SD rats by collagenase digestion of the pancreas and density gradient centrifugation using discontinuous Ficoll™ PM400 (Amersham Biosciences AB, Uppsala, Sweden), and then handpicked [12, 19]. Prior to PEGylation, isolated islets were cultured for three days in the RPMI-1640 (Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS; Sigma) at 37℃ in a humidified 5% CO_2 atmosphere. The purity of isolated islets was measured by dithizone staining (purity > 95%). Monomethoxy-PEG-succinimidyl carboxymethyl (mPEG-SCM, mw 5,000) was purchased from Laysan bio Inc. (Arab, AL). The isolated islets were first washed with Hank's balanced salt solution (HBSS) and then incubated for 1 h in HBSS (pH 8.0) containing 0.25% w/v mPEG-SCM for the chemical conjugation of PEG onto the collagen surface of isolated islets. Finally, the PEGylated islets were washed twice using RPMI-1640 and cultured in a culture medium for a day.

Fluorescein isothiocyanate (FITC) linked mPEG-SCM (FITC-PEG-SCM) was also immobilized onto the islets to evaluate the coverage of mPEG-SCM grafted onto the islet surface. FITC intensity was measured using a confocal laser scanning microscope (LSM510, Carl Zeiss, Germany), and

the intensity profile was obtained using the software (LSM Image Browser).

5.2.3. Sensitization of diabetic rats with PEGylated islets

Experimental diabetes was induced in inbred F344 rats by a single intraperitoneal injection of streptozocin (60 mg/kg; Sigma). Diabetes was defined as the state of non-fasting blood glucose level (NBG) above 350 mg/dl for 3 consecutive days. The blood glucose level was measured from blood withdrawn from a tail vein using a portable Gluco-card (Super glucocard II, Arkray, Kyoto, Japan).

For the preparation of sensitized splenocytes against PEGylated islets, PEGylated islets were transplanted into the diabetic F344 rats that had been anaesthetized by intraperitoneal injection with 50 mg pentobarbital/kg. The left kidney of the recipient was exposed through a lumbar incision and capsulotomy was performed on the caudal outer surface of the left kidney, and PEGylated islets were injected (3000 islets/recipient). Ten days after islet allotransplantation, the spleen was harvested from the recipients, pricked with a needle, and smoothly perfused with 20 ml of HBSS to make a suspension of splenocytes. Then the isolated splenocytes were incubated in Tris-buffered NH_4Cl solution (pH 7.2) for 5 min at room temperature for the lysis of red blood cells. The viability of splenocytes was verified, which was expected to be above 95%. For the preparation of non-sensitized splenocytes, the spleen of diabetic F344 rats was used without PEGylated islet allotransplantation.

5.2.4. Co-culture of PEGylated islets with splenocytes or sensitized splenocytes

The isolated splenocytes or sensitized splenocytes (1×10^5 cells/well) were co-cultured with 30 PEGylated islets in 200 μl of RPMI-1640 containing 10% FBS in 96-well flat-bottomed cell culture plates (Coring Co., NY). Both splenocytes and islets were cultured together for 9 days at 37℃ under humidified atmosphere containing 5% of CO_2. The culture medium (100 μl) was replaced every 2 days with a new culture medium.

The viability of PEGylated islet was determined by lactate dehydrogenase (LDH) assay and AO/PI staining, using fluorometric acridine orange (AO, Sigma) for inclusion staining and propidium iodide (PI, Sigma) for exclusion staining. Briefly, 100 μl of culture medium supernatant was obtained at each culture time (Day 1, 3, 5 and 7) after co-culture of PEGylated islets with splenocytes. Then LDH from the damaged islets was measured from the supernatant of culture medium using a LDH assay kit (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega). For AO/PI staining, viable cells (green color) were stained with AO, whereas dead cells (red color) were stained with PI. At each culture time, the co-cultured islets were washed with HBSS and re-suspended in 1 ml of HBSS containing 0.67 μM of AO and 75 μM of PI in a microtube. The islets were incubated at room temperature for 4 min in the dark. After triple washing, the islets were inspected with a fluorescence microscope (Eclipse TE2000S, Nikon, Japan).

To confirm the insulin secretion level, glucose stimulated insulin

secretion (GSIS) assay was performed using PEGylated islets co-cultured with sensitized and non-sensitized splenocytes for 7 days. Briefly, 50 islets were plated into a 24-well culture plate containing Millicell cultured plate inserts (Millipore). The PEGylated islets were pre-incubated for 1 h in 1 ml of Krebs-Ringer bicarbonate buffer (KRBB; pH 7.4) containing low glucose solution (2.8 mM). Following pre-incubation, the PEGylated islets were cultured with KRBB solution containing low glucose solution (2.8 mM) and high glucose solution (28 mM) for 2 h, respectively. The amount of secreted insulin from the PEGylated islets was measured using a rat/mouse insulin ELISA kit (Millipore). The stimulation index (SI) was calculated by dividing the amount of secreted insulin at high glucose solution by that of low glucose solution.

5.2.5. Repeated allotransplantation of unmodified and PEGylated islets with immunosuppressants

After transplanted islets were rejected, islets were repeatedly transplanted in the same rats. Briefly, PEGylated islets (3000 islets/recipient) were transplanted on the outer caudal surface of the left kidney. Seven days after the first allotransplantation without administering CsA, the left kidney having PEGylated islets was nephrectomized and new PEGylated islets (3000 islets/recipient) were transplanted in the right kidney subcapsule. For one of the two groups, 3 mg/kg of CsA was intravenously administered daily. On the other hand, for the other group, 3 mg/kg of CsA was intravenously

administered daily together with the intraperitoneal administration of 2 mg/rat of anti-CD4 mAb (at -1, 0, 1, 3, 5, 7 days of transplantation). For the control group, PEGylated islets were transplanted once in the left kidney and 3 mg/kg of CsA was daily administered for 30 days. In addition, to confirm the effect of PEGylation in combination with a low dose of immunosuppressive drug, the left kidney having unmodified islets was nephrectomized and new unmodified islets (3000 islets/recipient) were transplanted into the right kidney subcapsule seven days after the first round of unmodified islet transplantation without CsA administration. Following the transplantation, 2 mg/rat of anti-CD4 mAb (at -1, 0, 1, 3, 5, 7 days of transplantation) were administered daily into the recipients together with the intravenous administration of CsA (3 mg/kg/day) as shown in Fig. 5.1. Transplantation was considered successful if the blood glucose level of the recipients returned to the normal glucose level (<120 mg/dl) and was retained for two consecutive days after the transplantation. Islet rejection was diagnosed when the blood glucose level of recipients was retained over 200 mg/dl for two consecutive days.

5.2.6. Immunohistochemistry

The kidney in which islets were transplanted were fixed in 4% paraformaldehyde-PBS, embedded in paraffin, and cut into 4 μm thickness sections, followed by deparaffinizing with xylem and rehydrating through gradient ethanol immersion. Any endogenous peroxidase activities were

stopped by a peroxidase-blocking agent (0.3%, H_2O_2), followed by washing with distilled water.

Immunohistochemical staining for insulin, somatostatin, glucagon, $CD20^+$, $CD4^+$ and $CD8a^+$ were performed at room temperature for 1 h using primary antibodies: insulin antibody (1:50; ABcam Inc., Cambridge, MA), somatostatin antibody (1:50; Biomeda Corp., Burlingame, CA), glucagon antibody (1:100; ABcam Inc.), CD20 antibody (1:40; Santa Cruz Biotechnology Inc., Santa Cruz, CA), $CD4^+$ antibody (1:1000; ABcam Inc.), $CD8a^+$ antibody (1:100; BioLegend). The prepared sections were washed twice by using a washing buffer and then treated with the secondary antibody at room temperature for 30 min. The reaction products were visualized with DAB at room temperature for 2 min. The sections were counter-stained with hematoxylin for 30 sec and washed with water, and then mounted with Permount on coverslips. Immunohisochemistry images were obtained under a light microscope.

5.2.7. Statistical analysis

Survival times of transplanted islets were expressed as median ± SEM. Cytotoxicity and insulin secretion were expressed as mean ± SD. Statistical analysis was performed using the nonparametric *t*-test. A *p*-value of <0.05 was considered statistically significant.

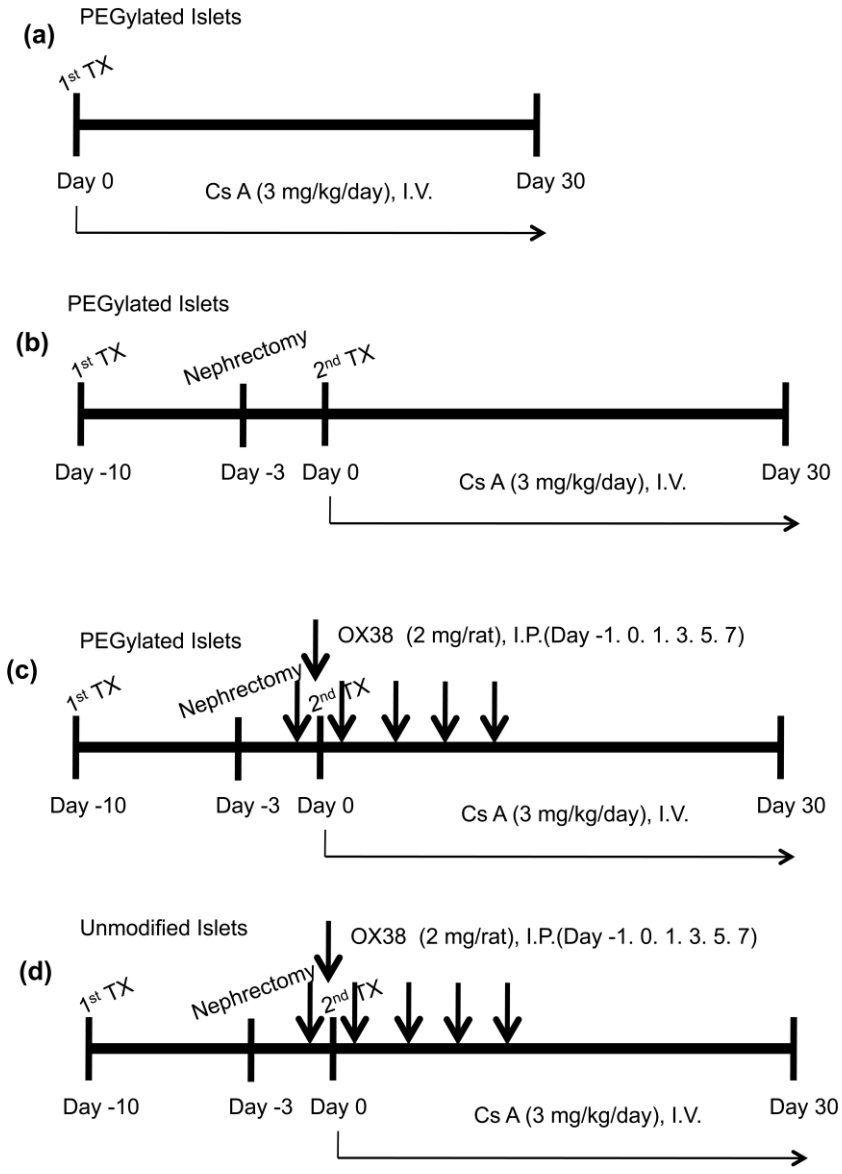

Figure 5.1. Experimental schedule for the first PEGylated islet transplantation with low dose of cyclosporine A (a) and the second batch of PEGylated islets with the administration of CsA (b) and CsA and anti-CD4 mAb (OX-38) (c) after being challenged with the first PEGylated islets without CsA. The second batch of unmodified islets with the administrations of CsA and anti-

CD4 mAb after being challenged with the first unmodified islets without CsA (d). The left kidney containing the first transplanted PEGylated or unmodified islets were nephrectomized before the second PEGylated or unmodified islets transplantation under the right kidney.

5.3. Results

5.3.1. PEGylation onto the islet surface

FITC-PEG-SCM was evenly distributed on the surface of islets, as shown in Fig. 5.2. Both PEG grafting time and PEG concentration were optimized in order to prevent any unnecessary diffusion of PEG molecules into the islets. The optimal PEG concentration was 0.25% (w/v) and PEG incubation time was 1 h in the previous study [11]. The fluorescence intensity of projection image indicated that the majority of islet surface was covered with FITC-PEG-SCM.

5.3.2. Co-culture of PEGylated islets with splenocytes *in vitro*

When PEGylated islets were cultured with splenocytes for 9 days, their viability was rarely damaged. However, the viability of PEGylated islets was severely damaged when they were cultured with sensitized splenocytes prepared from F344 rats that had PEGylated islets transplanted without any immunosuppressant *in vivo*. Fluorometric AO/PI stain results showed that PEGylated islets existed mostly as live cells (green color) even though they were cultured with splenocytes for 3 days. On the contrary, after PEGylated islets were cultured with sensitized splenocytes for 3 days, many of them were observed as dead cells (red color) (Fig. 5.3a). To quantitatively evaluate the viability of PEGylated islets cultured with splenocytes for 9 days, the secreted amount of LDH from the damaged PEGylated islets in the culture media was measured using the LDH assay kit. Compared to the PEGylated islets cultured

with splenocytes, the PEGylated islets cultured with the sensitized splenocytes showed a significantly increased amount of LDH (Fig. 5.3b). With the increased culture time, the difference in the amount of secreted LDH between the two groups mentioned above was also increased. After the co-culture of PEGylated islets with splenocytes for 1, 3, 5 and 7 days, the secreted amount of LDH was 0.15 ± 0.01, 0.26 ± 0.02, 0.63 ± 0.05 and 0.85 ± 0.03, respectively. However, after the co-culture of PEGylated islets with the sensitized splenocytes for 1, 3, 5 and 7 days, the secreted amount of LDH was 0.27 ± 0.02, 0.60 ± 0.07, 1.09 ± 0.07 and 1.45 ± 0.11, respectively. The LDH secreted from PEGylated islets co-cultured with sensitized splenocytes was statistically higher than that secreted from the PEGylated islets co-cultured with non-sensitized splenocytes.

The insulin secretion profiles of PEGylated islets co-cultured with sensitized and non-sensitized splenocytes were not significantly different (Fig. 5.3c) from each other. In addition, the SI of the PEGylated islets co-cultured with sensitized splenocytes (2.3 ± 1.2) was not statistically different from the PEGylated islets co-cultured with non-sensitized splenocytes (3.5 ± 2.3) (Fig. 5.3d) either. These results demonstrated that sensitized splenocytes did not significantly affect the functionality of PEGylated islets.

5.3.3. Allotransplantation of PEGylated islets in sensitized diabetic rats

To evaluate the immunoprotection effect of PEGylation on the islets

of repeated transplantation, PEGylated islets were transplanted twice: that is, a second transplantation was carried out in the sensitized diabetic rats that had been challenged in advance with PEGylated islets in the first transplantation without immunosuppressant CsA. When PEGylated islets were transplanted first in the diabetic rats with a continuous administration of a low dose of CsA (3 mg/kg/day), the blood glucose levels of all recipients were maintained in a normal range without any fluctuation (Fig. 5.4a). However, after the second allotransplantation of PEGylated islets into the diabetic recipients that had been sensitized with PEGylated islets in advance without CsA, the PEGylated islets were completely rejected within 10 days despite the continuous administration of CsA (Fig. 5.4b). The median survival time of PEGylated islets of the second transplantation was only 6.0 ± 0.5 days. However, when the PEGylated islets were accompanied by the combination therapy using CsA and anti-CD4 mAb in the second transplantation, their viability was significantly improved such that three out of five recipients showed the normal blood glucose level up to 30 days after transplantation (Fig. 5.4c). To confirm the PEGylation effect, the unmodified islets were transplanted into the recipients for the second time, following the administration of CsA and anti-CD4 mAb. Unmodified islets were all rejected within 20 days post-transplantation (Fig. 5.4d).

Therefore, although a repeated transplantation of PEGylated islets with the monotherapy of CsA could not cure the type 1 diabetic rat, a repeated transplantation of PEGylated islets accompanied by the combined therapy of CsA and anti-CD4 mAb could effectively prevent the sensitized immune

response and prolong the graft survival time (Fig. 5.4e).

5.3.4. Immunohistochemical analysis

Seven days after the allotransplantation of PEGylated islets accompanied by a low dose of CsA, the left kidney containing the islets was harvested and immunostained to observe the expression of hormones such as insulin and glucagon. The transplanted islets were observed to be stable in the transplanted site with insulin, glucagon and somatostatin well expressed (data not shown). On the other hand, when the right kidney containing PEGylated islets of the second transplantation with CsA was harvested from the sensitized rats and immunohistochemically analyzed, there were rare detections of anti-glucagon, anti-insulin, anti-somatostatin positive cells in the transplanted site (Fig. 5.5). The right kidney containing the PEGylated islets accompanied by the CsA and anti-CD4 mAb combination therapy was harvested after the second trial of transplantation in the sensitized rats and immunohistochemically analyzed (Fig. 5.5). The results revealed that mass of unmodified islet was not retained and the expressions of glucagon, insulin, or somatostatin proteins were rare.

To confirm the immune cell migration to the transplanted site, $CD4^+$, $CD8^+$, and $CD20^+$ positive cells were stained with antibodies. As a result, the host B cells were found on the periphery of islets during the first and second trials without infiltration into the islets. When the right kidney containing PEGylated islets of the second transplantation with CsA was harvested from

the sensitized rats and immunohistochemically analyzed, there were rare detections of anti-CD4, anti-CD8, or andti-CD20 cells in the transplanted site. In contrast, lots of anti-CD4 and anti-CD8 positive cells were recruited near the transplanted site, thereby rejecting the unmodified islets quickly compared to PEGylated islets (Fig. 5.6).

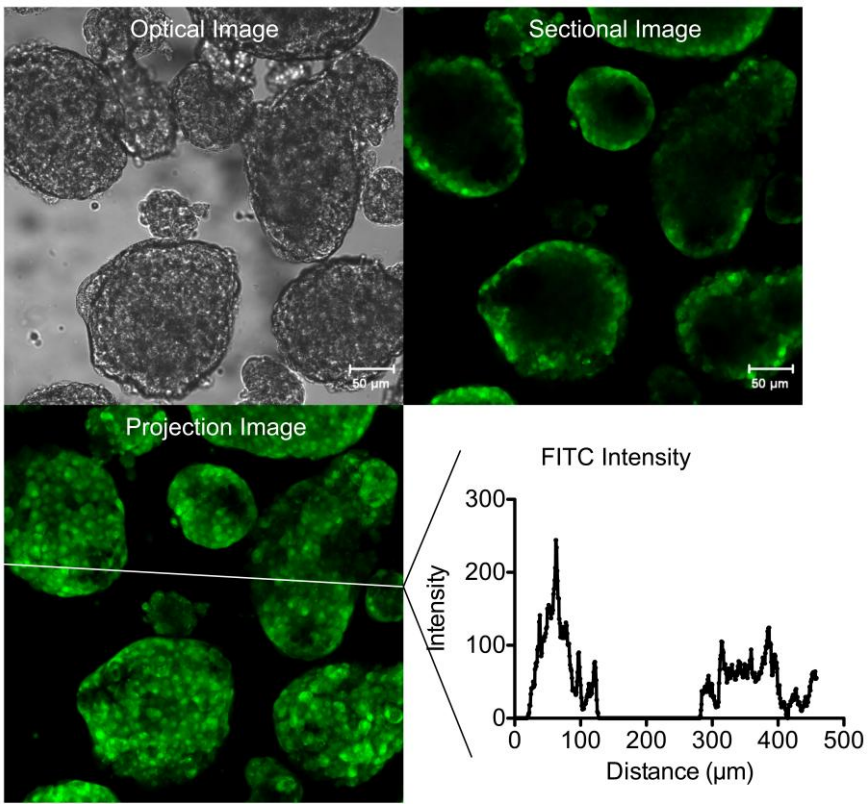

Figure 5.2. Confocal Laser Scanning Microscopic images of FITC-linked PEG-SCM grafted islets and the fluorescent intensity in comparison with the dark area.

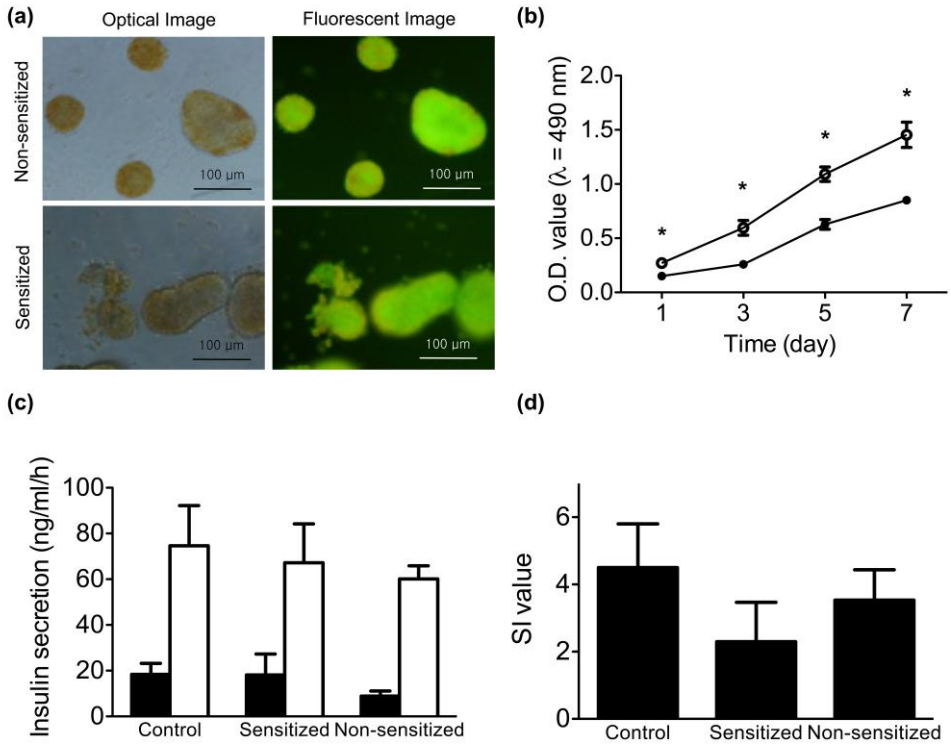

Figure 5.3. (a) Morphology and AO/PI stained fluorescent images of PEGylated islets cultured with splenocytes and sensitized splenocytes after 3-day co-culture. (b) The viability of PEGylated islets after co-culture with splenocytes (●) or sensitized splenocytes (○). Data were presented as mean ± SD, (n= 4). *P < 0.01 (c) The glucose stimulated-insulin secretion (GSIS) of PEGylated islets co-cultured with sensitized or non-sensitized splenocytes at low or high glucose solution for 2 h and (d) the Stimulation index (SI). Data were expressed as mean ± SD (n=5).

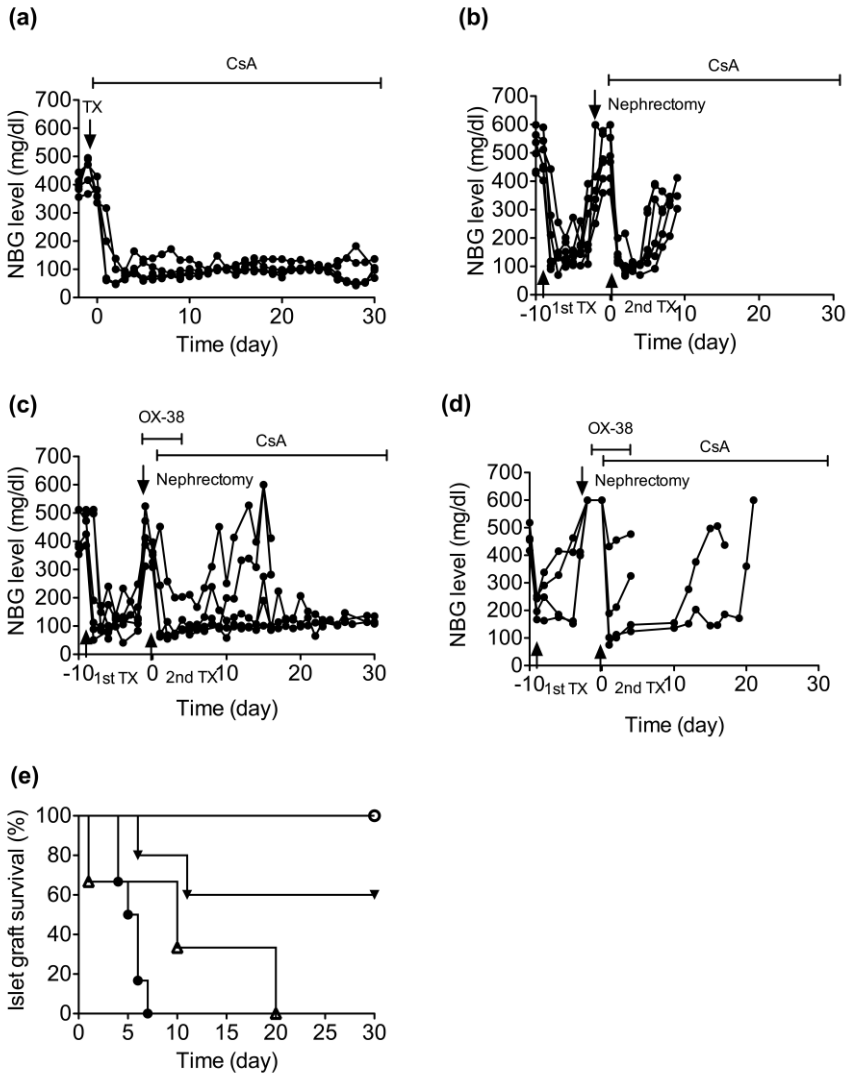

Figure 5.4. NBG levels of (a) non-sensitized recipients (n=5) were transplanted with PEGylated islets with a low dose of CsA. NBG levels of sensitized recipients were transplanted with PEGylated islets with the administration of (b) a low dose of CsA (n=6) or (c) a low dose of CsA and anti-CD4 mAb (OX-38) (n=5). (d) NBG of sensitized recipients transplanted with unmodified islets with the administration of low doses of CsA and anti-CD4 mAb (n=4). Sensitized recipients were prepared by PEGylated or

unmodified islet transplantation without CsA. CsA (cyclosporine A; 3 mg/kg/day) was daily i.v. injected via tail vein for 30 days. Anti-CD4 mAb (rat anti-CD4 monoclonal antibody; 2 mg/rat) was i.p. injected at -1, 0, 1, 3, 5, 7 days of transplantation. (e) Survival rates of PEGylated islets transplanted for the first time in non-sensitized recipients (○) with a low dose of CsA (3 mg/kg/day) (n=5), (●) sensitized recipients (n=6) when accompanied with or (▼) sensitized recipients (n=5) when accompanied with a low dose of CsA (3 mg/kg/day) plus anti-CD4 mAb (2 mg/rat). Survival rates of unmodified islets (△) in sensitized recipients after first round of transplantation when accompanied with a low dose of CsA (3 mg/kg/day) plus anti-CD4 mAb (2 mg/rat) (n=4).

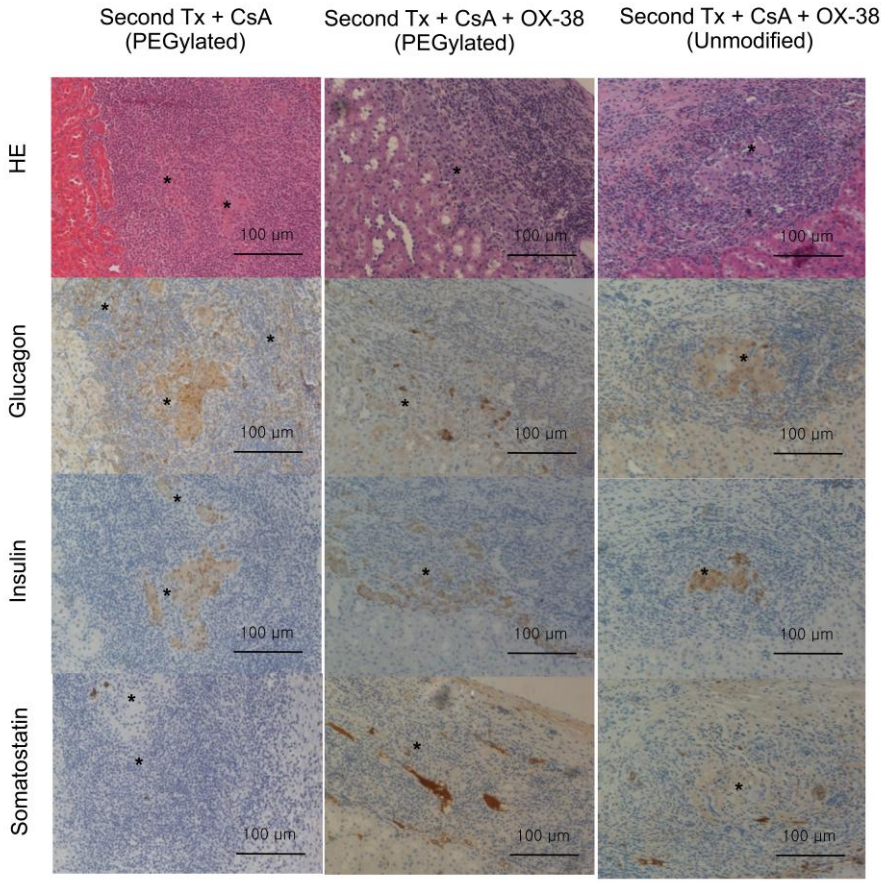

Figure 5.5. Immunohistochemical analysis of the second transplantations of PEGylated islets with the administration of CsA and PEGylated or unmodified islets with the combination therapy of CsA and anti-CD4 mAb (OX-38) in sensitized recipients 7 days after allotransplantation. Asterisk: PEGylated or unmodified islets. (HE; Hematoxylin and eosin stain)

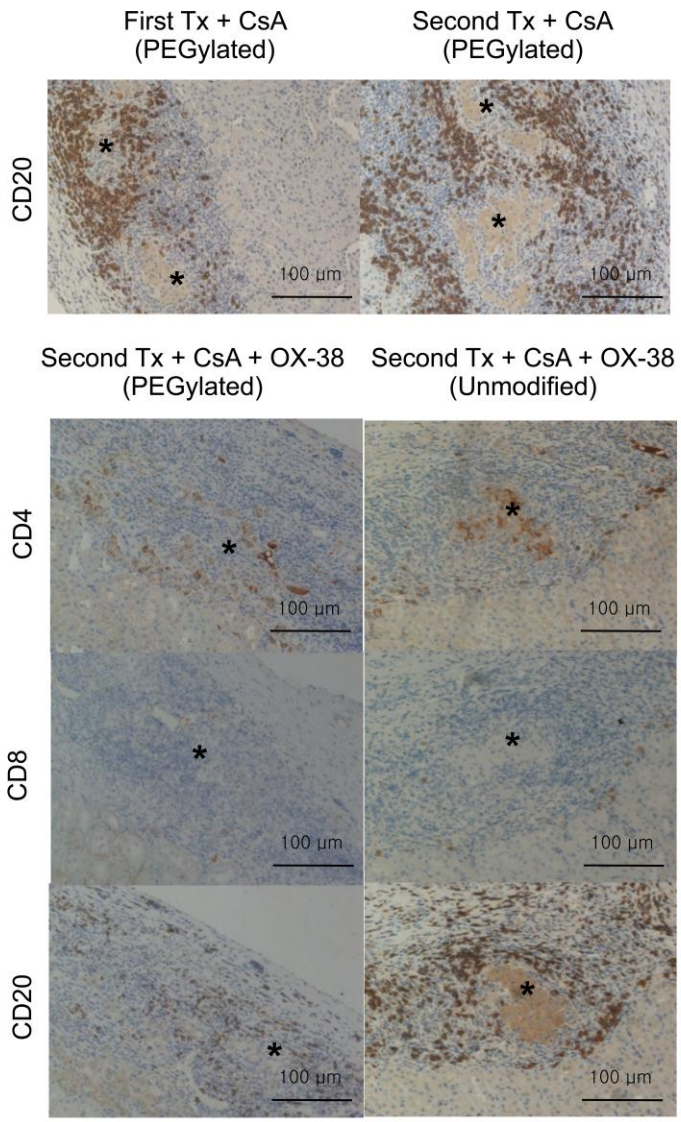

Figure 5.6. Immunohistochemical analysis of the first and second transplantions of PEGylated islets with CsA (CD20; B cell), and the second transplantion of PEGylated or unmodified islet with CsA and anti-CD4 mAb (OX-38) combination therapy (CD20; B cell, CD4 and CD8a; T cell) in the sensitized recipients 7 days after allotransplantation, respectively. Asterisk: PEGylated or unmodified islets.

5.4. Discussion

We showed in this study that a second trial of PEGylated islet allotransplantation in diabetic rats, which had been previously sensitized with PEGylated islets from the same species of rats without CsA, resulted in an accelerated rejection of transplanted PEGylated islets although a low dose of CsA was continuously administered. The accelerated rejection of PEGylated islets was not only enhanced by the sensitization with islets from a MHC-mismatched strain within the same species, i.e., allotransplantation, but also by the secreted antigens from islets. Immunohistochemical results showed that there were no immune cell infiltrations in neither of the PEGylated islets of the first and second trials because the conjugated PEG molecules on the surface of islets can physically block the infiltration of immune cells. Furthermore, PEGylated islets of the first transplantation in the non-sensitized recipients were well stained with insulin, glucagon and somatostatin antibody in the transplanted site, whereas those of the second trial transplanted in the sensitized recipients were rarely stained with insulin, glucagon and somatostatin antibody although CsA was continuously injected into the recipients. These results indicated that protein expression of the PEGylated islets in the second transplantation in the sensitized rats was damaged by cytokines induced by the enhanced graft rejection although the islets still existed in the transplantation site. Secretion of insulin by islets is known to be affected by cytotoxic cytokines such as IL-1β and TNF-α [20, 21]. Also, our previous study demonstrated that the conjugated PEG molecules on the

surface of islets could not protect the infiltration of cytotoxic cytokines into islets even though PEGylated islets blocked the infiltration of immune cells into the islets [10]. Thus, it was possible that memory B-cells and T-cells in the sensitized recipients rapidly recognized the PEGylated islets of the second transplantation and secreted large amounts of cytokines and antibodies even though they could not be infiltrated into the PEGylated islets.

Although combined therapy of PEGylation and a low dose of CsA could well protect the transplanted islets in non-sensitized rats, it was difficult for this combined therapy to block the cytotoxic cytokine and antigen-antibody interaction in sensitized rats. In fact, serological results of the second trial of islet transplantation in the sensitized rats indicate that the eosinophil level was higher than that of the first islet transplantation in the non-sensitized rats although a low dose of CsA was continuously administered in both rat groups. This result might be attributed to the production of interleukin-5 (IL-5) cytokine induced by interacting B-cells with Th2 T cells [22]. Furthermore, while naive $CD4^+$ T cells primarily produced IL-2 shortly after TCR triggering, memory $CD4^+$ T cells were able to rapidly produce effectors associated cytokines upon re-stimulation [23]. Therefore, when treated with only a low dose of CsA, the second trial of PEGylated islets could not be effectively prevented from memory $CD4^+$ T cells-mediated accelerating immune response.

To increase the survival time of PEGylated islets of the repeated transplantation for preventing of the sensitized immune rejection, combinatory treatments of low doses of CsA and anti-CD4 mAb, which

abbreviated the activation of indirect immune destruction triggered by migrated sensitized antigens, were needed to synergistically enhance the PEGylation effect on repeatedly transplanted islets. On the other hand, the graft survival time of unmodified islets in the repeated transplantation was not improved even with the treatment of low dose of CsA and anti-CD4 mAb. These findings suggested that islet PEGylation remedy could synergistically improve the viability and function of the repeated islet transplantation when treated with a combination therapy of CsA and anti-CD4 mAb.

5.5. Conclusion

We developed a combined therapy using PEGylation and immunosuppressant administration that immunologically improved islet survival after repeated allotransplantation. PEGylation of islet surface very effectively blocked the infiltration of host immune cells in both non-sensitized and sensitized recipients. However, PEGylation itself was not enough to protect the transplanted islets because of the significantly accelerated production of cytokines and antibodies induced by memory immune cells. Therefore, other immunosuppressive protocols are needed for repetitive PEGylated islet allotransplantation in sensitized recipients; in particular, it would be important to suppress the activation of memory $CD4^+$ T cells triggered by indirect immune response

5.6. References

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Chapter 6. Conclusion

In this study, many strategies have been investigated to solve the obstacles associated with islet transplanted. To enhance the functionality of beta cell, secretion signal peptide linked exendin-4 gene was transduced into islets for solving the islet donor shortage. Insulin secretion ability of LV-SP-Ex-4 transduced islets was around three fold higher than untransduced islets. Surprisingly, the transduction of LV-SP-Ex-4 gene did not affect the viability of islets. In diabetic animal model, only 50 LV-SP-Ex-4 transduced islets were needed to be transplanted in order to cure the diabetic nude mice, however, at least 150 transduced islets needed to achieve normoglycemia. Therefore, the highly releasable exendin-4 could enhance the beta cell function and can be presented as a potential strategy to overcome islet donor shortage.

Attenuation of the immune was considered as one of the major barrier in islet transplantation, thus biocompatible polymer (6-arm-PEG-catechol) was suggested as a potent immunoprotective polymer to block the immune cells activation. 6-arm-PEG-catechol was used for coverage of islets surface and it has effectively regulated the blood glucose level of recipients for more than 50 days following transplantation and administration of low dose of immunosuppressive drugs (Anti-CD154 mAb and tacrolimus). Capability of insulin secretion of surface camouflaged islets was preserved after transplantation and was evaluated using IPGTT. In addition, the number of migrated immune cells was significantly reduced in the area of grafted islets

Therefore, newly developed combinatory immunoprotection protocol of surface camouflaged islets with a low dose of immunosuppressive drugs can be concluded as an effective strategy to prolong the graft survival rate of

islets in xenotransplantation. Furthermore, these kinds of polymer therapy will be applied to prevent immune reaction in porcine and human islet transplantation.

In addition, the severity of complication related to type 2 diabetes such as liver toxicity, insulin resistance and fatty liver was reduced after surface camouflaged islet transplantation in type 2 diabetic mouse model. The blood glucose level of type 2 diabetic mice with 6-arm-PEG-catechol grafted islets reversed hyperglycemia. Even though the graft survival time of islets did not increase dramatically, the blood glucose level of type 2 diabetic *db/db* mouse model reached normoglycemia after islet transplantation. In addition, the proposed islet transplantation protocol inhibited the pathophysiological deterioration of type 2 diabetes during that time by way of reducing islet size expansion in the pancreas, preventing adipocyte size expansion and fat accumulation in the liver. This proposed study is still under improvement. To enhance the survival time of grafted islets in type 2 diabetes, genetic engineering or protein delivery could be used to improve insulin sensitivity and to alleviate insulin resistance.

Finally, to evaluate the immunoprotection effect of PEGylated islets in repeated transplantation to prevent sensitized immune rejection, combinatory treatments of low doses of cyclosporin A (CsA) and anti-CD4 mAb (OX-38) were used to synergistically enhance the PEGylation effect on repeatedly transplanted islets. The graft survival time of repetitively grafted PEGylated islets was significantly improved when compared to the unmodified islets treated with a combination of CsA and anti-CD4 mAb.

Therefore, we have concluded that islet PEGylation could synergistically improve the viability and functionality of islets in repeated transplantation when used in combination with CsA and anti-CD4 mAb.

Collectively, these studies suggested the new therapeutic method for the treatment of diabetes mellitus using genetically engineered and surface camouflaged islets can be suggested as clinically applicable strategy to treat diabetes mellitus. In future perspective, more studies on developing potent immunoprotection protocol must be completed in order to treat diabetes mellitus in clinic. I hope that this study will enhance current knowledge regarding immunomodulatory therapy and function of genetically engineered islets in diabetes mellitus treatment.

요약

당뇨병 치료를 위한 표면 개질 및 유전자 조작 췌장소도의 이식

정 지 헌

서울대학교 대학원

약학대학

제약학과 물리약학 전공

제 1 형 당뇨병 (type 1 diabetes mellitus) 의 일반적인 치료 방법은 인슐린을 주사하는 것이다. 하지만 인슐린 주사의 경우 저혈당 쇼크 (hypoglycemic shock)가 유발된 가능성 이 높고, 주사 부위의 알레르기 (allergy) 반응, 그리고 당뇨병에 의한 합병증 (complication)의 유발을 막기에는 한계가 있다. 따라서, 최근에 근본적인 제 1 형 당뇨병 치료를 위한 방법으로 췌도세포 이식 방법이 활발히 연구 되고 있다. 하지만 췌도세포 이식의 경우에도 성공적인 치료가 되기 위해서 다양한 문제점이 존재 하고 있는데, 특히 이식 시 발생하는 면역 반응은 가장 큰 장벽 중 하나로 여겨진다.

이와 같은 문제점을 해결하기 위해서 현재 임상에서는 여러 가지 면역 억제제 투여를 통해 면역 활성화를 억제할 수 있는 에드몬톤 프로토콜 (Edmonton Protocol)방법이 쓰여지고 있다. 하지, 인슐린 비의존도의 비율이 1년 까지는 90%에 이르지만 5년이 지나면 50% 도 되지 않고 있다. 따라서 명백히 한계점을 가지고 있다고 생각된다. 다시 말하면 새로운 방법 면역 억제 치료법의 개발의 필요가 절실한 시점이다. 그리고 또 하나의 큰 문제점은 췌도세포 공급의 해결이다. 약 2-3 명의 공여자로부터 췌도세포를 분리하여야 이식을 통해서 한 명의 당뇨병 환자를 치료할 수 있기 때문이다. 최근에는 다양한 실험적 방법을 통해 분리하는 췌도세포의 생존율을 향상 시켜 최소한의 췌도세포 이식으로 치료할 수 있는 연구가 되고 있지만 아직 까지는 한계점이 있는 실정이다. 따라서 많은 연구자들은 돼지 췌도세포를 이용한 타종간의 이식이나 줄기세포를 이용한 접근을 많이 연구 하고 있다. 하지만 이러한 방법의 경우 임상적용까지의 시간이 많이 요구 되고 있는 것이 현실이다. 따라서 본 연구에서는 인슐린의 분비능의 향상을 유도하는 유전자의 전달을 통해 근본적인 세포 공급문제 해결에 접근 하였다. 제 2 형 당뇨병의 치료제로 사용하고 있는 엑센딘-4(Exendin-4) 유전자를 렌티바이러스(Lenti-viral vector)를 이용하여 전달 하였다. 그리고 세포 밖으로의 분비를 극대화 하기 위해 분비 신호 펩타이드(secretion signal peptide)까지 연결을 해 놓은 유전자를 전달 하였다. 그 결과 인슐

린 분비능의 약 3배 이상 향상 되었고, 누드 마우스 당뇨모델에 이식하자 오직 50개의 유전자 전달 췌도세포 만으로도 혈당 조절이 30일 이상 지속 되었으며 (대조군은 150개의 췌도세포가 필요했음), 정상적인 면역체계를 가지고 있는 C57BL/6 마우스에 유전자가 전달된 췌도세포를 이식할 경우에도 생존율이 현저히 증가함도 확인하였다. 따라서, 엑센딘-4 유전자의 전달은 현재 췌도세포 이식의 문제점 중 하나인 세포 공급문제의 하나의 해결책으로 제안할 수 있었다.

그리고, 앞에서 언급한 또 하나의 문제점인, 면역 반응을 최소화 하기 위해서 생체 적합성 고분자 물질인 분지형 폴리에틸렌글라이콜 (6-arm-PEG-catechol)을 이용하여 표면을 개질 하였다. 기존의 선형 폴리에틸글라이콜을 이용하 방법에 비해서 복층으로 췌도세포 표면을 개질하여 더 강력한 타종간의 이식 반응 까지도 막을 수 있었다. 표면 개질 기술은 췌도세포의 생존율 및 기능성에는 큰 영향을 미치지 않을 뿐 아니라 표면 개질도 결점 없이 완전히 이루어 졌다. 동물실험에서는, 표면 개질 된 췌도세포를 이식하고 낮은 농도의 면역 억제제 (Anti-CD154 mAb; 0.1 mg/mouse, 이식일 기준 0, 2, 4, 6 일째 와 tacrolimus; 0.2 mg/kg/day)을 동시에 투여하자, 면역 억제의 시너지 효과가 발생하여 모든 당뇨모델 마우스가 정상 혈당을 50일 이상 유지 하는 것을 확인하였다. 그러므로, 기존의 면역억제제의 한계점을 생체적합성 고분자의 추가적인 치료

를 통해 면역 억제제의 용량을 줄임으로써 약 부작용의 최소화 시킬뿐 아니라 췌도세포의 생존율에도 큰 향상을 가져오는 방법으로 제안할 수 있었다.

이식한 췌도세포의 경우 시간이 지남에 따라 면역 반응에 의해 거부 반응이 일어나게 되므로 추가적인 인슐린 치료나 췌도세포의 재이식이 요구된다. 이때 한번 췌도세포 이식에 의해 면역 반응이 활성화 되어 있기 때문에 감작된 면역 반응(sensitized immune reaction)은 강한 면역활성화로 인해 더 쉽게 췌도세포의 거부 반응이 일어날 수 있다. 본 연구에서는 췌도세포 표면 개질 기술을 이용하여 재이식시 발생하는 감작된 면역 반응에도 충분한 면역 억제효과를 가질 수 있는 지를 확였다. 선형 폴리에틸렌클라이콜로 표면 개질을 한 후 랫트 당뇨병 모델에 이식을 하였고 약물 Anti-CD4 mAb (OX-38, 2 mg/rat, 이식일 기준 -1, 0, 1, 3, 5, 6 일째) 및 사이클로스포린 (cyclosporin A, 3 mg/kg/day)를 투여 하였다. 이때 5마리중 3마리는 30일 이상 정상 혈당을 유지했지만, 표면 개질 하지 않은 췌도세포의 경우는 모두 거부반응이 발생했다. 즉, 췌도세포 표면개질 기술이 감작된 면역 반응에서도 억제하는 효과가 있다는 것을 확인 하였다.

마지막으로 이러한 표면 개질 기술을 이용한 치료 프로토콜이 제 2 형 당뇨 모델에서도 적용가능한지를 평가하였다. 제 2 형 당뇨병의 경우도 인슐린의 치료를 도입할 경우 다양한 부작용의 감

소 및 비용절감 그리고 마지막 단계에서는 반듯이 필요한 방법으로 이식되고 있다. 따라서 인슐린 투여를 대신하여 면역 억제 프로토콜을 이용하여 췌도세포를 이식하여 효과를 평가 하였다. 제 2 형 *db/db* 마우스 모델에 표면 개질한 췌도세포를 이식을 하고 면역 억제제를 투여하자, 이식한 췌도세포는 2주일간 제 2 형 당뇨모델 쥐의 혈당을 정상으로 유지 시켰으며, 간독성 및 췌장의 췌도세포 확장, 비만세포의 크기 등의 억제 효과를 보였다. 오랜 기간 동안의 혈중 농도 조절이 이루어 지지는 않았지만 제 2 형 당뇨의 진행을 현격히 감소 시키는 효과 가 있었으며 유전자 및 단백질 전달 치료를 병행 한다면 더 좋은 결과를 얻을 수 있을 것으로 여겨 진다.

본 연구를 통해 표면 개질 기술 및 유전자 조작 기술을 췌도세포 이식에 적용하여 랫트 와 마우스 당뇨병모델 효과적으로 치료할 수 있었다. 더 나아가서는 돼지 및 인간 췌도세포에 적용하여 임상에 적용할 수 있을 것으로 기대된다.

주요어: 당뇨병, 췌장소도 이식, 표면 개질, 유전자 조작, 폴리에틸렌 글라이콜, 엑센딘-4

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