Protective Strategies for Enhancing Engraftment of Insulin Releasing Cells

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CONTENTS

ABBREVIATIONS

APC	Antigen-presenting cell
Biotin-PEG-NHS	Biotin-poly(ethylene glycol)-N-hydroxysuccinimide ester
BSA	Bovine serum albumin
DA	Degree of angle
DMSO	Dimethyl sulfoxide
DPPE	1,2-dipalmitoyl-*sn*-glycerol-3-phosphatidylethanolamine
DSPE	1,2-distearoyl-*sn*-glycerol-3-phosphatidylethanolamine
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EGFP	Enhanced green fluorescent protein
EggPC	L-*α*-phosphatidylcholine Type XVI-E from egg yolk
ELISA	Enzyme-linked immunosorbent assay
ES cell	Embryonic stem cell
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
HBSS	Hanks' balanced salt solution
H_2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
HE	Hematoxylin-eosin
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HUVEC	Human umbilical vein endothelial cell
IBMIR	Instant blood-mediated inflammatory reaction
IgG	Immunoglobulin G

IPGTT	Intraperitoneal glucose tolerance test
iPS cell	Induced pluripotent stem cell
Islet	Islet of Langerhans
KRB	Krebs–Ringer's buffer
MACS	Magnetic-activated cell sorting
2-ME	2-mercaptethanol
MSC	Mesenchymal stem cell
MW	Molecular weight
NBD-lipid	1-Palmitoyl-2-[12-[(7-nitro-2-1, 3-benzoxadiazol-4-yl)amino] dodecanoyl]-*sn*-glycero-3-phosphoethanolamine
NHS-PEG-Mal	*α*-*N*-Hydroxysuccinimidyl-*ω*-maleimidyl poly(ethylene glycol)
NK	Natural killer
Oligo$(dA)_{20}$	20-mer of deoxyadenylic acid
Oligo$(dT)_{20}$	20-mer of deoxythymidylic acid
PBS	Phosphate-buffered saline
PBS(+)	Phosphate-buffered saline containing 0.9 mM calcium chloride and 0.33 mM magnesium chloride
PEG	Poly(ethylene glycol)
ROS	Reactive oxygen specie
SPR	Surface plasmon resonance
ssDNA	Single-stranded oligonucleotides
STZ	Streptozotocin
Sulfo-EMCS	N-[*ε*-maleimidocaproyloxy] sulfosuccinimide ester
TEA	Triethylamine

TGF	Transforming growth factor
Treg cell	Regulatory T cell
TRITC	Tetrarhodamine isothiocyanate
Tween 20	Polyoxyethylene sorbitan monolaurate
Type I diabetes	Insulin-dependent diabetes mellitus
UK	Urokinase
UW	University of Wisconsin
Vitamin E	DL-α-tocopherol

GENERAL INTRODUCTION

Cell or organ transplantation[1–5] has attracted much attention as a promising method of treating serious diseases. Various kinds of pluripotent stem cells, such as embryonic stem (ES) cells, induced pluripotent stem (iPS) cells, and mesenchymal stem cells (MSCs), have been developed or identified,[6–10] and their differentiation to functional cells[11–15] and formation of a three-dimensional tissue[16–19] have been extensively studied. However, their clinical application remains limited because contact between pluripotent stem cells and living tissues and blood induces unfavorable recipient reactions, including graft rejection,[20–22] autoimmune disease recurrence,[23,24] and ischemia-reperfusion damage[25,26] (Figure 1). For example, the administration of immunosuppressive drugs[27] is required to inhibit immune reactions against allogeneic cells or organs and control autoimmune reactions against iPS-derived cells. The effects

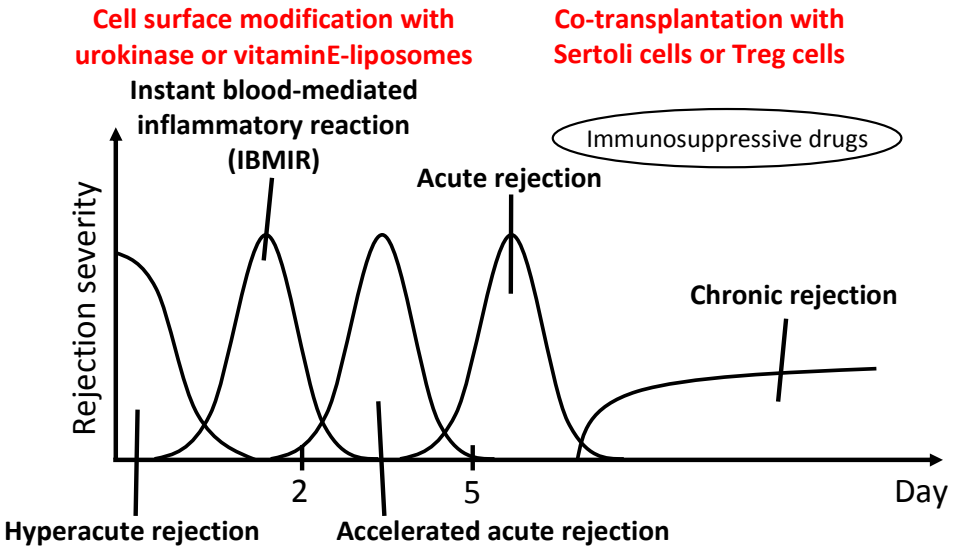


Figure 1. Unfavorable recipient reactions in cell or organ transplantation and protective strategies to improve graft survival.

of the long-term use of immunosuppressants on recipients are not fully understood.[28–30] Methods for cell or organ transplantation therapy without the use of immunosuppressive drugs are desired. Two approaches are being explored to overcome these issues: surface modification[31–35] and co-aggregation.[16,36–38]

The modification of the surface of these cells or organs is expected to be useful in their transplantation and reduce unfavorable recipient reactions. Modification of the surfaces of living cells with polymeric materials also allows for new opportunities in biomedical engineering and science because a variety of functional groups and bioactive substances can been introduced onto the cell surface, adding new functionality to the cell.The co-aggregation of these cells and immune-related cells, such as MSCs,[9,39,40] Sertoli cells,[41–43] and regulatory T (Treg) cells,[44–46] is also believed to play an active role in the creation of an immunosuppressive environment at the engraftment site, which is considered the optimal location for immunologic graft protection. Because the effect would be focused locally, the immune modulation would be stronger (and therefore safer and more precise). In addition, the methods employed to achieve the co-aggregation of different cell types allow for new opportunities in biomedical engineering and science because most cells derived from pluripotent stem cells do not function appropriately without cooperating closely with other cell types.[47] Cell surface modifications and co-aggregations can each be applied to the study of various biomedical topics, such as development, regenerative medicine, and the immune system, some of which will be discussed here in some detail.

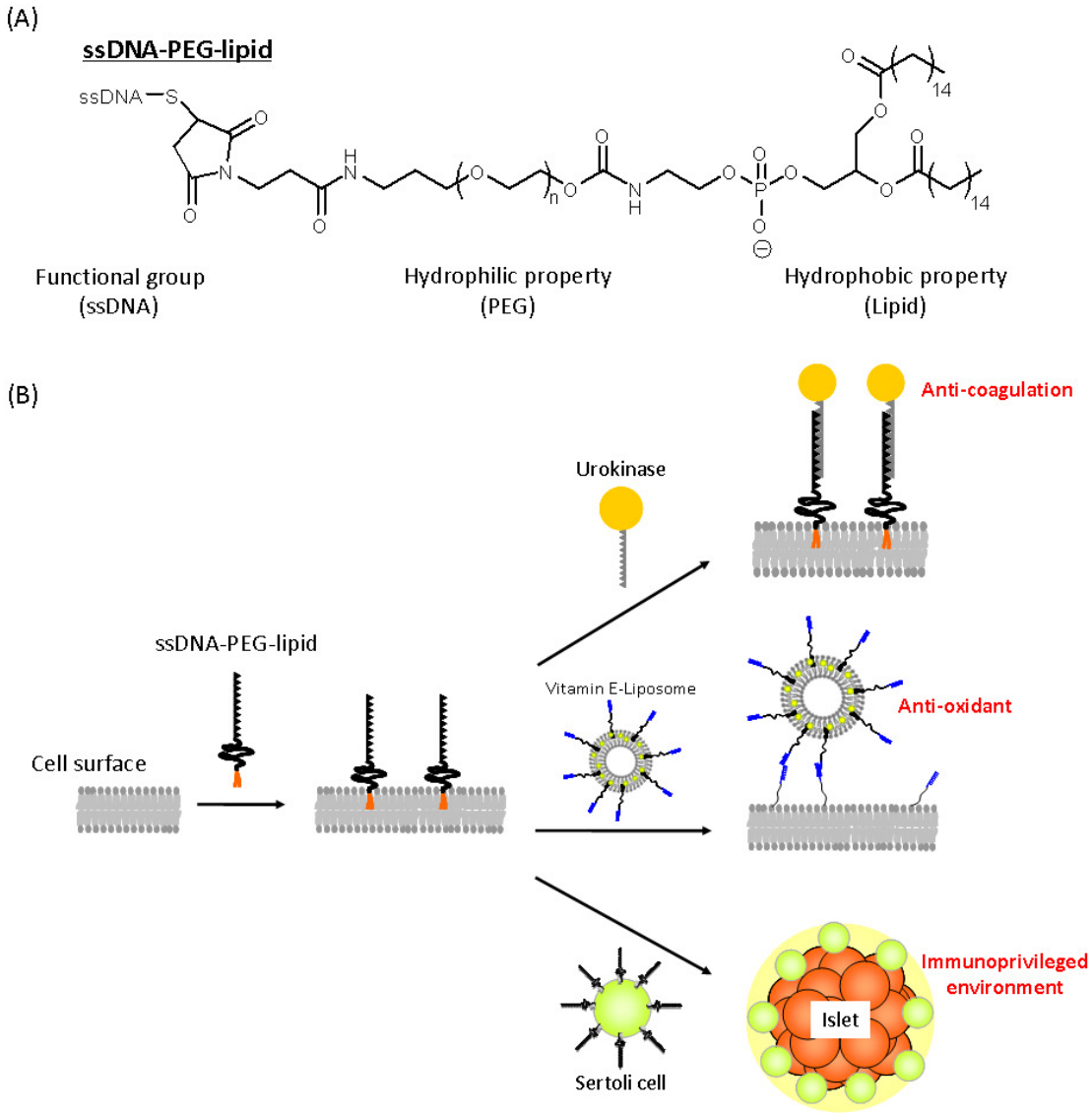
The aim of this thesis is to elucidate the effects of surface modification and co-aggregation on cell/organ transplantation. This thesis consists of the following two parts, which are subdivided into a total of five chapters.

Part I is concerned with cell surface modification (Scheme 1). Chapter 1 describes the surface modification of islets of Langerhans (islets)[48] with the fibrinolytic enzyme urokinase (UK)[49] through DNA hybridization. Islet transplantation has been proposed as a safe, effective approach to treating patients with insulin-dependent diabetes mellitus (type I diabetes).[1,50] However, many islets are reportedly lost during the early phase after intraportal transplantation as a result of instant blood coagulation-mediated inflammatory reactions.[22,51,52] In this chapter, DNA hybridization was applied to conjugate UK[49] to the islet surface. Amphiphilic polymers, poly(ethylene glycol)-phospholipid (PEG-lipid)-carrying 20-mers of deoxythymidylic acid ($oligo(dT)_{20}$) ($oligo(dT)_{20}$−PEG-lipid; PEG MW = 5000)[35] and UK-carrying 20-mers of complementary deoxyadenylic acid ($oligo(dA)_{20}$) were synthesized. The $oligo(dT)_{20}$−PEG-lipid was spontaneously incorporated into the cell membrane through interactions between the hydrophobic parts of the PEG-lipid and the lipid bilayer,[35] and UK was conjugated to the cell surface through DNA hybridization between $oligo(dT)_{20}$ on the cell and complementary $oligo(dA)_{20}$ on the UK. The activity of UK was maintained on the islet surface. Modification of the surface with UK did not influence islet morphology or islet ability to secrete insulin in response to changes in glucose concentration. No practical volume increase was observed with our method, indicating that islet graft loss could be suppressed at the early stage of intraportal islet transplantation.

Chapter 2 describes the introduction of antioxidant-loaded liposomes into endothelial cell surfaces through DNA hybridization. Ischemia-reperfusion damage is a problem in organ transplantation.[25,26] Blood-mediated reactions produce reactive oxygen species at the time of blood reperfusion.[25,26,53] This chapter describes the

development of a method to immobilize and internalize antioxidants in endothelial cells using vitamin E-loaded liposomes.[54,55] Liposomes loaded with vitamin E and human umbilical vein endothelial cells (HUVECs) were modified with PEG-lipid conjugates carrying oligo$(dA)_{20}$ and complementary oligo$(dT)_{20}$, respectively. The liposomes were effectively immobilized on HUVECs through DNA hybridization between oligo$(dA)_{20}$ and oligo$(dT)_{20}$. The vitamin E-loaded liposomes were gradually internalized into the HUVECs. Next, the HUVECs were treated with antimycin A[56] to induce oxidative stress. The amount of reactive oxygen species was greatly reduced in HUVECs carrying vitamin E-loaded liposomes.

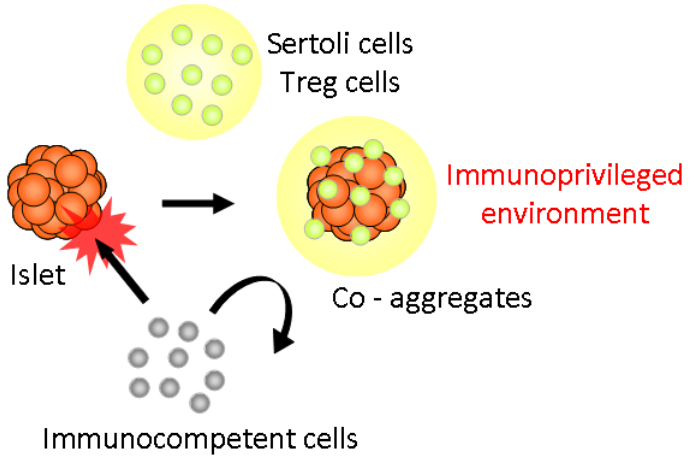
Chapter 3 describes the immobilization of Sertoli cells[41–43] on islets. Sertoli cells play a crucial role in creating the immunoprivileged environment of the testis.[57] The survival of islets after co-transplantation with Sertoli cells was examined, with the hypothesis that Sertoli cells near islets should protect the graft from rejection.[58,59] In this chapter, single-stranded oligonucleotides conjugated to PEG-lipids (ssDNA−PEG-DPPE)[35] were used to immobilize Sertoli cells on islets. Oligo$(dA)_{20}$ and complementary oligo$(dT)_{20}$ were presented as ssDNAs on the surfaces of Sertoli cells and islets, respectively, through a hydrophobic interaction between a lipid unit of the conjugate (PEG-DPPE) and the cell membrane.[35] The Sertoli cells were immobilized on the islets by hybridizing oligo$(dA)_{20}$ (present on Sertoli cells) with oligo$(dT)_{20}$ (present on islets). When Sertoli cell-immobilized islets were infused into the livers of mice through the portal vein, the Sertoli cells remained around the islets.



Scheme 1. Cell surface modifications. (A) The chemical structure of single-stranded oligonucleotide and poly(ethylene glycol)-phospholipid conjugates (ssDNA−PEG-lipids). (B) Cell surface modifications with proteins, liposomes, or other cells using ssDNA−PEG-lipids.

Part II is concerned with co-aggregation (Scheme 2). Islet transplantation has been used to treat type I diabetes.[1,50] However, islet grafts must be maintained with the administration of immunosuppressive drugs, which can lead to complications in the long term.[30] An approach that avoids immunosuppressive drug use is desirable. Chapter

4 describes the transplantation of co-aggregates of Sertoli cells[41–43] and islet cells into liver without immunosuppression. Co-aggregates of Sertoli cells and islet cells from BALB/c mice were prepared using the hanging drop method and transplanted into C57BL/6 mouse liver through the portal vein as in human clinical islet transplantation. The co-aggregate core contained mainly Sertoli cells, and these cells were surrounded by islet cells. The co-aggregates retained the functions of both Sertoli and islet cells. When 800 co-aggregates were transplanted into C57BL/6 mice via the portal vein (n=7), six of the seven recipient mice demonstrated quasi-normoglycemia for >100 days. The hanging drop method is suitable for preparing aggregates of Sertoli and islet cells for transplantation. Notably, transplantation of these allogeneic co-aggregates into mice with chemically induced diabetes via the portal vein resulted in long-term graft survival without systemic immunosuppression.



Scheme 2. Co-aggregation of islets of Langerhans (islets) with Sertoli cells or regulatory T (Treg) cells to improve graft survival.

Chapter 5 describes the transplantation of co-aggregates of $CD4^+CD25^+$ Treg cells[44–46] and islet cells. These co-aggregates also allow long-term graft survival in liver without immunosuppression. Co-aggregates of Treg cells from C57BL/6 mice and islet cells from BALB/c mice were prepared on agarose hydrogel with small round-bottomed wells. Treg cells and islet cells were randomly distributed in the co-aggregates. Four hundred co-aggregates were transplanted into the livers of streptozotocin-induced diabetic C57BL/6 mice (n=9) without systemic immunosuppression. Six of the nine recipients demonstrated quasi-normoglycemia for >100 days. A number of insulin-positive cells were observed in the livers at 120 days post-transplantation.

In summary, this thesis describes the use of cell surface modification and co-aggregation methods to enhance therapeutic cell or organ engraftment. It is anticipated that these technologies, still an emerging research area, will be applicable to the treatment of patients in the areas of regenerative medicine, tissue engineering, stem cell research, and embryology.

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PART I

CELL SURFACE MODIFICATION

Chapter 1

Islet Surface Modification with Urokinase through DNA Hybridization

INTRODUCTION

Transplantation of islets of Langerhans (islets) has been proposed as a safe and effective method for treating patients with insulin-dependent diabetes mellitus (type I diabetes).[1,2] Intraportal islet transplantation combined with the Edmonton protocol has had demonstrable clinical success. Multiple donors of pancreases, however, are still needed to achieve normalized blood glucose levels without insulin injection. This necessity is attributable to the loss of many islets in the early phase after intraportal transplantation by instant blood-mediated inflammatory reactions (IBMIR).[2–8] It has been reported that a lot of islets are known to be damaged by IBMIR within a few hours.[9] If blood coagulation induced by transplanted islets can be inhibited, most of the islets could be rescued.

Systemic administration of some anticoagulants has been tried to achieve this outcome,[6,7] but patients may then be at a high risk for bleeding. Recently, alternative ideas have been proposed for the suppression of IBMIR,[10–18] including islets coated with anticoagulants such as heparin,[10] thrombomodulin,[11] fibrinolytic urokinase (UK),[14,15] or living cells,[12,16,18] to localize their antithrombogenic effects. Various improvements are still needed, however, for clinical application of these approaches. In

some studies, the biotin/streptavidin reaction has been used to conjugate antithrombogenic materials. Streptavidin, however, is an immunogenic protein derived from bacteria[19,20] and is likely not appropriate for use in surface modification of islets for clinical uses. In the other studies, chemical modification of membrane proteins has been employed, but this method is expected to deteriorate islet cell functions.

Iwata and colleagues have synthesized various amphiphilic polymers and applied them to surface modification of cells to conjugate antithrombogenic materials and modulate the immunogenicity of cells.[13–18,21–26] Poly(ethylene glycol)-phospholipid conjugates (PEG-lipids) have been successfully applied to cell surface modification. They are spontaneously incorporated into the cell membrane through interactions between the hydrophobic parts of the PEG-lipids and the lipid bilayer. Iwata and colleagues have further advanced their technology to easily modify the cell surface with various bioactive substances by conjugating single-stranded DNA with PEG-lipids (ssDNA–PEG-lipids).[18,24] In this chapter, DNA hybridization were employed to modify the islet surface with the plasminogen activator UK using ssDNA–PEG-lipid.

EXPERIMENTAL PROCEDURES

Materials.

α-*N*-Hydroxysuccinimidyl-*ω*-maleimidyl poly(ethylene glycol) (NHS-PEG-Mal, MW 5000), 1,2-dipalmitoyl-*sn*-glycerol-3-phosphatidylethanolamine (DPPE), and 1,2-distearoyl-*sn*-glycerol-3-phosphatidylethanolamine (DSPE) were purchased from NOF Corporation (Tokyo, Japan). Dichloromethane, chloroform, diethyl ether, dimethyl

sulfoxide (DMSO), triethylamine (TEA), penicillin-streptomycin mixed solution, and heparin sodium salt were bought from Nacalai Tesque (Kyoto, Japan). Fetal bovine serum (FBS) was purchased from BioWest (Miami, FL, USA) and phosphate-buffered saline (PBS) from Nissui Pharmaceutical, Co., Ltd. (Tokyo, Japan). Alexa 488-labeled rabbit antigoat IgG, HEPES buffer solution, Medium 199, and Hanks' balanced salt solution (HBSS) were purchased from Invitrogen, Co. (Carlsbad, CA, USA). Urokinase (UK) was purchased from American Research Products, Inc. (Belmont, MA, USA), polyclonal goat antihuman UK from Abcam plc. (Cambridge, UK), and N-[ε-maleimidocaproyloxy] sulfosuccinimide ester (Sulfo-EMCS) from Pierce Biotechnology, Inc. (Rockford, IL). Thrombin was obtained from Kayaku Co., Ltd. (Saitama, Japan). Fibrinogen type I, plasminogen (from human plasma), and oligo$(dA)_{20}$ and oligo$(dT)_{20}$ carrying a protected SH group at the 5'-end (oligo$(dA)_{20}$-SH, oligo$(dT)_{20}$-SH) were purchased from Sigma-Aldrich Chemical Co. (St. Louis,MO,USA). Oligo$(dA)_{20}$ and oligo$(dT)_{20}$ with a 5'-end SH group were prepared by reduction of the disulfide bond with dithiothreitol (DTT) according to the manufacturer's instructions.

Synthesis of Oligo$(dT)_{20}$−PEG-Lipids and Oligo$(dA)_{20}$-Conjugated UK.

Oligo$(dT)_{20}$-conjugated PEG-lipids (oligo$(dT)_{20}$–PEG-DPPE, oligo$(dT)_{20}$–PEG-DSPE, Scheme 1(a)) were synthesized following the method reported previously.[18,24] UK (1 mg/mL in PBS, 100 μL) and sulfo-EMCS (0.38 mg/mL in PBS, 20 μL) were mixed together and left for 2 hours at room temperature to prepare Mal-UK (Scheme 1(b)). Mal-UK was isolated with Sephadex G25 and freeze-dried. Mal-UK (1 mg/mL in PBS, 20 μL) was mixed with oligo$(dA)_{20}$-SH (150 μg/mL in PBS, 80 μL) for

2 hours at room temperature to prepare oligo$(dA)_{20}$-UK.

Surface Modification of Islets with UK through DNA Hybridization Using Oligo$(dT)_{20}$−PEG-Lipids.

All animal experiments were approved and accepted by the animal care committee of Institute for Frontier Medical Sciences, Kyoto University. Pancreatic islets were isolated from the pancreas of Syrian hamsters (7 weeks old, female, Japan SLC, Inc., Shizuoka, Japan) by the collagenase digestionmethod.[27] As shown in Scheme 1(c), oligo$(dT)_{20}$–PEG-lipid was conjugated with islets as reported previously.[18,24] Briefly, oligo$(dT)_{20}$–PEG-lipid (500 μg/mL oligo$(dT)_{20}$–PEG-DPPE in PBS or 500 μg/mL oligo$(dT)_{20}$–PEG-DSPE in PBS containing 10% DMSO) was added to an islets suspension and left for 1 hour at room temperature for oligo$(dT)_{20}$–PEG-DPPE or 30 minitues at 37 °C for oligo$(dT)_{20}$–PEG-DSPE. After the islets were washed with Medium 199 (serum free), oligo$(dA)_{20}$-UK (200 μg/mL) was added to the islets and left for 1 hour at room temperature.

Immunostaining for UK was carried out as follows: UK-islets were incubated with 2% polyclonal goat antihuman UK antibody for 1 hour at room temperature. After washing with medium, the UK-islets were incubated with fluorescence labeled secondary antibody and 0.2% Alexa 488-labeled rabbit antigoat IgG in medium for 1 hour at room temperature, followed by washed with medium. Those islets were observed under a confocal laser scanning microscope (FV500; Olympus, Tokyo, Japan).

Measurement of UK Activity with the Fibrin Plate Assay.

To examine the fibrinolytic activity of UK conjugated on islets, the fibrin plate

assay was employed as previously reported with slight modifications.[28] Briefly, 8 mL of fibrinogen solution (10 mg/mL in saline supplemented with 0.2 mg plasminogen) was poured into a culture dish (ø =10 cm). A total of 30 μL of thrombin (60 IU/mL) was added to the solution and mixed well by rotating the plate. The plate was incubated for 3 hours at room temperature to prepare a fibrin plate. The UK-islets (50 islets) cultured just after UK conjugation for 0 or 2 days and unmodified islets (50 islets) were applied to each spot on the fibrin plate. After incubation at 37 °C for 14 hours, the size of the transparent area formed around the islet spots was determined to measure fibrinolytic activity.

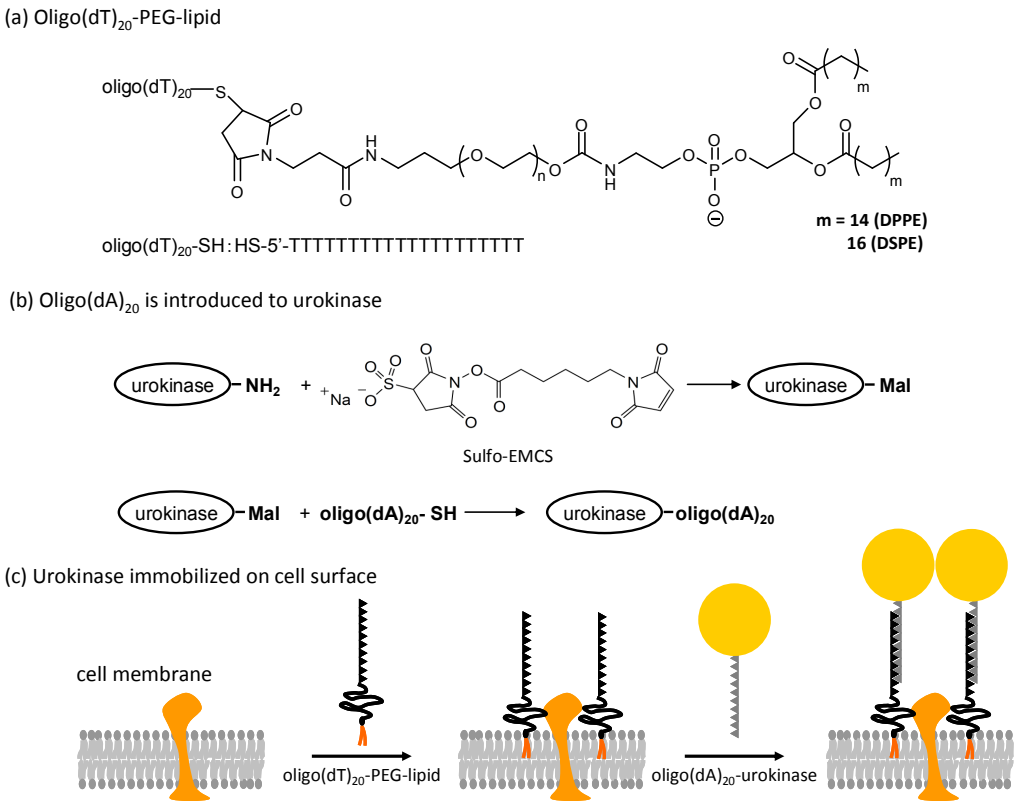
Glucose Stimulation Test of UK-Islets.

Static insulin secretion tests were performed using 50 islets to examine their ability to change insulin secretion in response to changes in glucose concentration. Unmodified islets and UK-islets after 0 or 2 days in culture were used for the assay. Islets were exposed to 0.1 g/dL, then 0.3 g/dL, and finally 0.1 g/dL of glucose in Krebs-Ringer solution for 1 hour each at 37 °C. The solutions were collected after the 1-hour incubation in each glucose concentration, and insulin concentrations were determined in each solution by enzyme-linked immunosorbent assay (ELISA). The ELISA kit for the insulin assay was purchased from Shibayagi, Co., Ltd. (Gunma, Japan).

RESULTS

UK Conjugation on Islet Surfaces through DNA Hybridization.

Scheme 1 shows the method for islet surface modification with UK through DNA hybridization using a ssDNA–PEG-lipid conjugate. When the oligo(dT)$_{20}$–PEG-lipid is mixed with islets, the hydrophobic lipid part is spontaneously incorporated into a lipid bilayer of the cell membrane through the hydrophobic interaction.[18,24] The oligo(dT)$_{20}$



Scheme 1. Method to Conjugate UK on the Cell Surface through DNA Hybridization. (a) Chemical structure of oligo(dT)$_{20}$-conjugated PEG-lipid (oligo(dT)$_{20}$–PEG-lipid). (b) UK is modified with Sulfo-EMCS to introduce maleimide groups, and then oligo(dA)$_{20}$-SH is conjugated on UK through the thiol/maleimide reaction. (c) Oligo(dT)$_{20}$–PEG-lipid is incorporated into the cell surface by the hydrophobic interaction between alkyl chains and the lipid bilayer of the cell membrane. Oligo(dA)$_{20}$-UK is applied to cells carrying oligo(dT)$_{20}$. UK is conjugated on the cell surface through oligo(dT)$_{20}$–oligo(dA)$_{20}$ hybridization.

segment of the oligo$(dT)_{20}$–PEG-lipid was presented on the cell surface. It can be used for the conjugation of bioactive substances on the cell surface through the hybridization of oligo$(dT)_{20}$–oligo$(dA)_{20}$.

Confocal Fluorescent Microscopic Observation of UK Conjugation on the Islet Surface.

Islets were sequentially treated with oligo$(dT)_{20}$–PEG-DPPE or oligo$(dT)_{20}$–PEG-DSPE and oligo$(dA)_{20}$-UK as illustrated in Scheme 1(c). Oligo$(dA)_{20}$-modified UKwas conjugated on a cell surface that had been modified with oligo$(dT)_{20}$–PEG-lipid. Figure 1 shows confocal laser-scanning fluorescence images of UK-islets, which were subjected to immunostaining for UK. Fluorescence was clearly observed on the UK-islets (Figure 1(a)). However, very weak fluorescence was observed on unmodified islets or islets treated with oligo$(dA)_{20}$-UK in the absence of oligo$(dT)_{20}$–PEG-lipids due to a nonspecific stain (Figure 1(b)). The conjugation of UK through DNA hybridization was also demonstrated on a gold surface carrying oligo$(dT)_{20}$ by surface plasmon resonance (SPR) measurement (see Figure S1 in Supporting Information). These results indicated that oligo$(dT)_{20}$ groups could be introduced onto the islet surface through the hydrophobic interaction between two long alkyl chains of the oligo$(dT)_{20}$–PEG-DPPE or oligo$(dT)_{20}$–PEG-DSPE and a lipid bilayer of the islet membrane, and oligo$(dA)_{20}$-UK could be conjugated through DNA hybridization onto the islet surface where oligo$(dT)_{20}$–PEG-lipids had been introduced.

The retention time of oligo$(dT)_{20}$–PEG-lipid on the cell membrane depends on the chain lengths of PEG-lipids.[26] Here, oligo$(dT)_{20}$–PEG-DPPE and oligo$(dT)_{20}$–PEG-DSPE with alkyl chains of 16 and 18 (–CH_2–) groups, respectively,

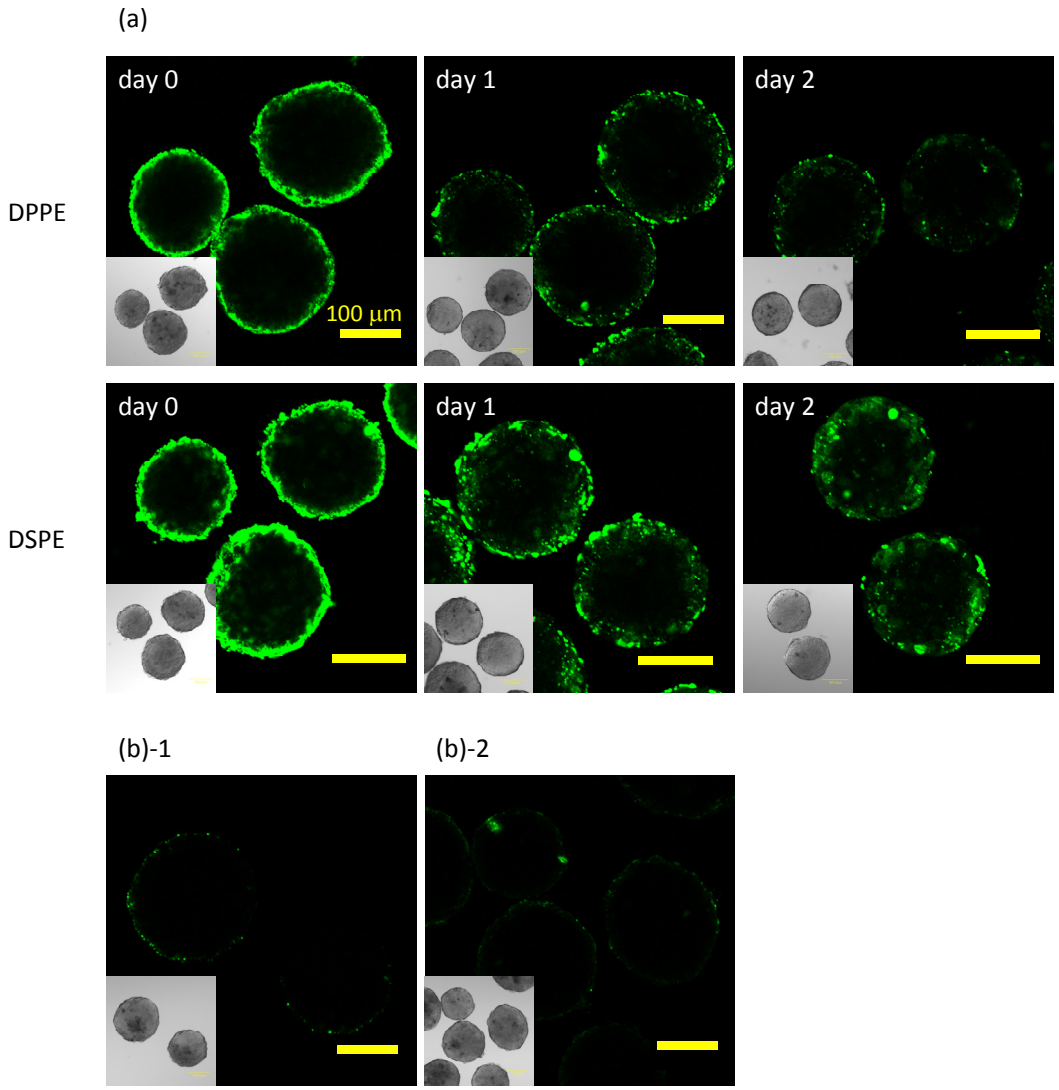


Figure 1. Confocal laser scanning microscopic images of UK-islets which were subjected to immunostaining for UK. (a) Islets were modified with oligo(dT)$_{20}$–PEG-DPPE or oligo(dT)$_{20}$–PEG-DSPE and then oligo(dA)$_{20}$-UK. The UK-islets just after treatment, after 1 and 2 days of culture. ((b)-1) Unmodified islets. ((b)-2) Islets without treatment with oligo(dT)$_{20}$–PEG-lipids. Insets: phase contrast microscopic image. Scale bar: 100 μm.

were employed to determine the effect of chain lengths on the retention time of UK on islets. Oligo(dA)$_{20}$-UK could be conjugated onto the islet surface via oligo(dT)$_{20}$–oligo(dA)$_{20}$ hybridization without damaging islet morphology (Figure 1a). For 2 day cultures after conjugation, fluorescence was still observed for islets treated

with oligo$(dT)_{20}$–PEG-DSPE, whereas fluorescence faded for those treated with oligo$(dT)_{20}$–PEG-DPPE. The retention of UK on the islet surface was prolonged with increasing alkyl chain length.

UK Conjugation onto the Islet Surface through DNA Hybridization.

UK is a serine protease that activates plasminogen into plasmin, which dissolves fibrin blood clots. UK was conjugated onto the surface of islets to dissolve blood clots surrounding islets in the liver for inhibition of the cascade reactions of IBMIR. Islets were treated with oligo$(dT)_{20}$–PEG-lipid (oligo$(dT)_{20}$–PEG-DPPE or oligo$(dT)_{20}$–PEG-DSPE) and then with oligo$(dA)_{20}$-UK as illustrated in Scheme 1(c). A fibrin plate–based assay was performed to assess the function of the UK conjugated on the islets. Fifty islets with/without UK were applied to each spot on a fibrin gel plate, and the area of dissolved fibrin formed around the spotted UK-islets was observed. Figure 2 shows the fibrin plate at 14 hours after spotting of the UK-islets. Larger transparent areas were observed around the UK-islets than were seen surround the unmodified islets, as shown in Figure 2a 1, 2, and 4. These indicate that the UK retained its activity after conjugation on the islets. Islets that were cultured for 2 days after UK conjugation were also applied to the fibrin plate for the determination of the retention of UK activity. The transparent areas for the islets carrying UK through oligo$(dT)_{20}$–PEG-DSPE (Figure 2a 5) were slightly larger than that for the islets carrying UK through oligo$(dT)_{20}$–PEG-DPPE (Figure 2a 3). The morphology of all islets after modification with UK was maintained well after 7 days of culture in medium (Figure 2b). These results indicated that the conjugation of UK by DNA hybridization did not deteriorate the islets.

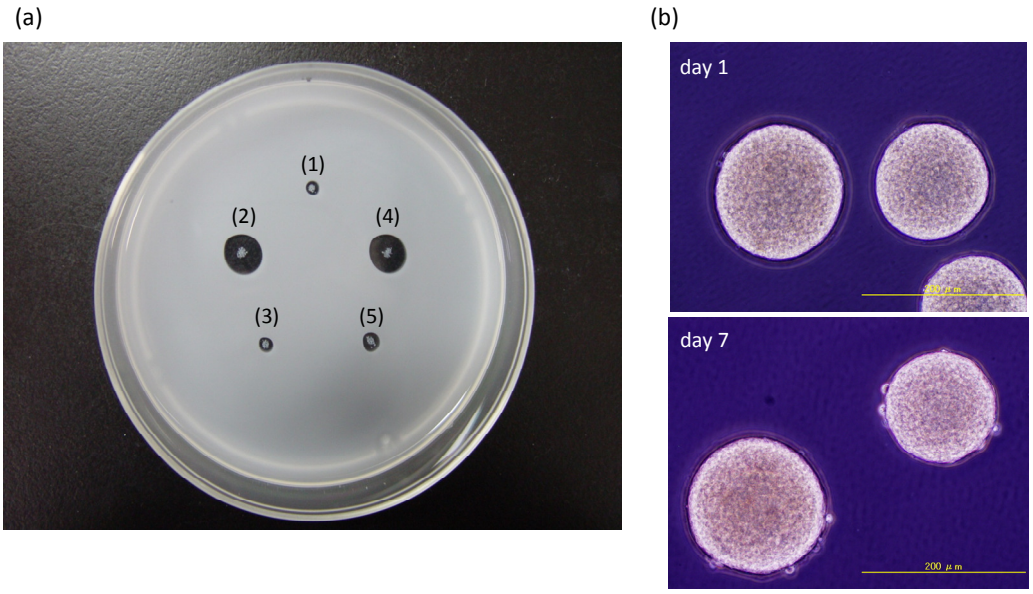


Figure 2. (a) Fibrin plate-based assay for UK-islets. (1) Unmodified islets; (2) UK conjugated on islets through oligo(dT)$_{20}$–PEG-DPPE just after preparation; (3) UK conjugated on islets through oligo(dT)$_{20}$–PEG-DPPE after 2 days of culture; (4) UK conjugated on islets through oligo(dT)$_{20}$–PEGDSPE just after preparation; and (5) UK conjugated on islets through oligo(dT)$_{20}$–PEG-DSPE after 2 days of culture. Fifty islets were applied to each spot, and the plate was observed after incubation at 37 °C for 14 hours. (b) Morphology of UK conjugated islets after 1 and 7 days of culture.

Glucose Stimulation Test of UK-Islets.

Static glucose stimulation tests were performed to evaluate the effect of UK conjugation on the insulin-releasing ability of islets. The tests were performed just after UK conjugation or after an additional 2 days of culture. Figure 3 summarizes the results. When the glucose concentration was increased from 0.1 g/dL to 0.3 g/dL, amounts of released insulin increased from basal levels for all groups. Although the amounts of released insulin from UK-islets were less than those from the unmodified islets, the glucose stimulation indices for modified islets were greater than those of the unmodified islets. The amounts of released insulin decreased to basal levels when the islets were re-exposed to low glucose (0.1 g/dL). Although the surface modification

with PEG-lipid and UK-conjugation slightly deteriorated the insulin secretion ability of islets, the modified islets controlled insulin release in response to glucose level changes.

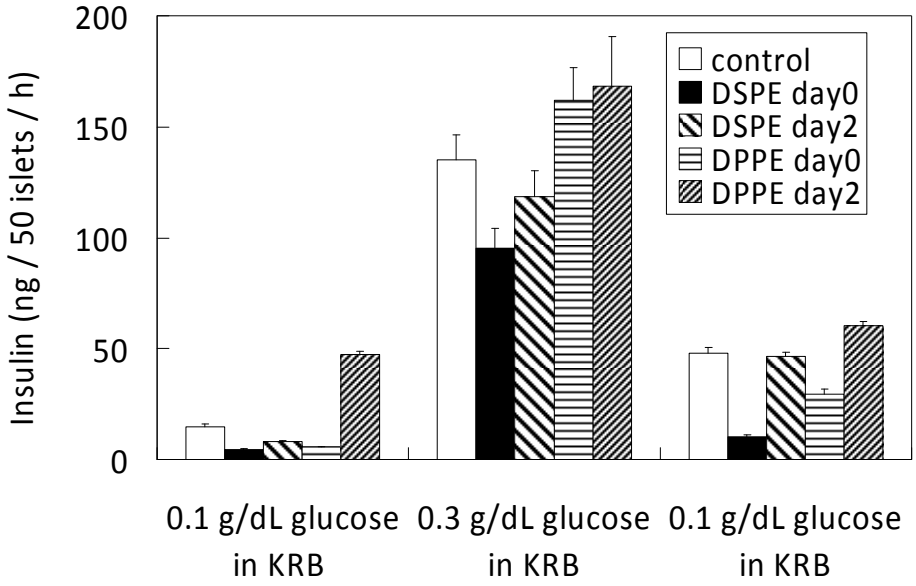


Figure 3. Glucose stimulation test. Bars from the left indicate amounts of insulin release for unmodified islets, UK-islets (oligo(dT)$_{20}$–PEG-DSPE) just after preparation, UK-islets (oligo(dT)$_{20}$–PEG-DSPE) after 2 days of culture, UK-islets (oligo(dT)$_{20}$–PEG-DPPE) just after preparation, and UK-islets (oligo(dT)$_{20}$–PEG-DPPE) after 2 days of culture.

DISCUSSION

Some obstacles remain for clinical islet transplantation, including the side effects of immune-suppressive drugs, low efficiency in islet isolation, difficulty of islet preservation, and early graft loss by IBMIR. With early graft loss induced by IBMIR, thrombotic reactions initiate when islets come into direct contact with blood in the portal vein. Blood coagulation triggers successive reactions, such as the inflammatory

response, infiltration of leukocytes into islets, disruption of islet integrity, and resulting islet loss.[2–8] Therefore, it has been thought that suppression of blood coagulation would lead to better graft survival. To improve the blood compatibility of islets, the islet surface has been coated with anticoagulants such as heparin and thrombomodulin, or fibrinolytic UK, and the living cells examined.[10–18] Iwata and colleagues have reported some methods to conjugate bioactive substances onto the surface of islets using amphiphilic polymers such as PEG-lipid through hydrophobic interactions.[13–16] PEG-lipid is anchored to the lipid bilayer of the cell membrane through hydrophobic interactions, and the functional group at the other end of the PEG chain can be used for further conjugation of bioactive substances. In one study, they employed the biotin/streptavidin reaction;[14] although this reaction is strong and specific, it is difficult to apply in clinical islet transplantation because of the strong antigenecity of streptavidin, which is derived from bacteria. They also examined the thiol/maleimide reaction in another study[15] and found that the conjugation efficiency requires improvement because thiol groups easily form disulfide bonds under physiological conditions, resulting in low efficiency. They have sought improved methods to overcome these issues.

Further, Iwata and colleagues have examined DNA hybridization to immobilize cells onto a surface. In a previous study,[24] oligo$(dT)_{20}$ carrying a thiol group was introduced onto a gold surface, and cells carrying oligo$(dA)_{20}$ were applied to the surface. The cells were locally immobilized on a specific site where oligo$(dT)_{20}$ was introduced. In this chapter, this method was applied to conjugate UK on islets. Amphiphilic polymers, PEG-lipids carrying ssDNA were synthesized. These were conjugated onto the cell membrane through interactions between the hydrophobic parts

of the PEG-lipids and the lipid bilayer, and UK was conjugated on the cell surface through DNA hybridization between oligo$(dT)_{20}$ on the cell and complementary oligo$(dA)_{20}$ on the UK. As shown in Figures 1, 2, and 3, no adverse effect was observed in terms of islet morphology and function. This DNA hybridization is a versatile method for the conjugation of various bioactive substances on the cell surface. ssDNAs rapidly form a complex under physiological conditions, and the complex is biocompatible. Moreover, it is expected that several different kinds of substances can be conjugated easily by using different DNA sequences.

The retention time of PEG-lipids on cell membranes depends on the alkyl chain length of lipids, and the retention time of PEG-DSPE on cell membranes is longer than that of PEG-DPPE.[26]

In this chapter, UK was expected to be retained longer on islets when oligo$(dT)_{20}$–PEG-DSPE was employed compared to when oligo$(dT)_{20}$–PEG-DPPE was employed; however, no clear prolongation was observed in UK activity, as shown in Figure 2a. UK activity disappeared during 4 days of culture in either case because the hydrophobicity of DSPE or DPPE was not great enough for the conjugation of a hydrophilic protein like UK with its high molecular weight (54 kDa). Thrombosis formation initiates when islets come into direct contact with blood in the portal vein, resulting in early graft loss induced by IBMIR. On the basis of these findings, it is expected that islets can be rescued by the conjugation of UK on the islet surface because UK controls thrombosis formation. The efficacy of the UK-islets in inhibiting IBMIR should be carefully examined using *in vivo* models.

Chapter 1

SUPPORTING INFORMATION

Surface Plasmon Resonance Analyses of UK Immobilization through DNA Hybridization.

To examine the conjugation of urokinase through DNA hybridization, an in-house–designed surface plasmon resonance (SPR) instrument[1] was used for monitoring the reaction. Gold-coated BK-7 glass plates (refractive index: 1.515, size: 25 × 25 × 1 mm, Arteglass Associates Co., Kyoto, Japan) were immersed in a solution of oligo$(dT)_{20}$-SH (50 μg/mL in PBS) for 24 hours to conjugate oligo$(dT)_{20}$ onto the gold surface through Au/thiol bonding. The glass plates were washed thoroughly with pure water and 2-propanol sequentially. Each glass plate was set in the SPR flow cell, and a blocking solution of BSA (10 mg/mL in PBS) flowed onto the surface, followed by flowing of a solution of oligo$(dA)_{20}$-UK (5 μg/mL in PBS). In a control experiment, a solution of oligo$(dT)_{20}$-UK (5 μg/mL in PBS) was applied after flowing of blocking solution. The sample solutions and PBS were allowed to circulate through the flow cell at 4.0 mL/min, and all SPR measurements were performed at 37 °C.

The SPR method, which serves to monitor events on the gold surface, was employed to examine binding and stability of proteins through the DNA hybridization between oligo$(dA)_{20}$-modified proteins and oligo$(dT)_{20}$ on the gold thin layer in real time. Figure S1 shows the SPR profiles for interactions between oligo$(dA)_{20}$-UK or oligo$(dT)_{20}$-UK and oligo$(dT)_{20}$ on the substrate. The initial increases in the SPR profiles show conjugation of oligo$(dT)_{20}$-SH on the gold surface by the Au/thiol reaction. The surface was blocked by a BSA solution for 10 minitues before the addition of a oligo$(dA)_{20}$-UK or oligo$(dT)_{20}$-UK solution. The second increase in the solid line of

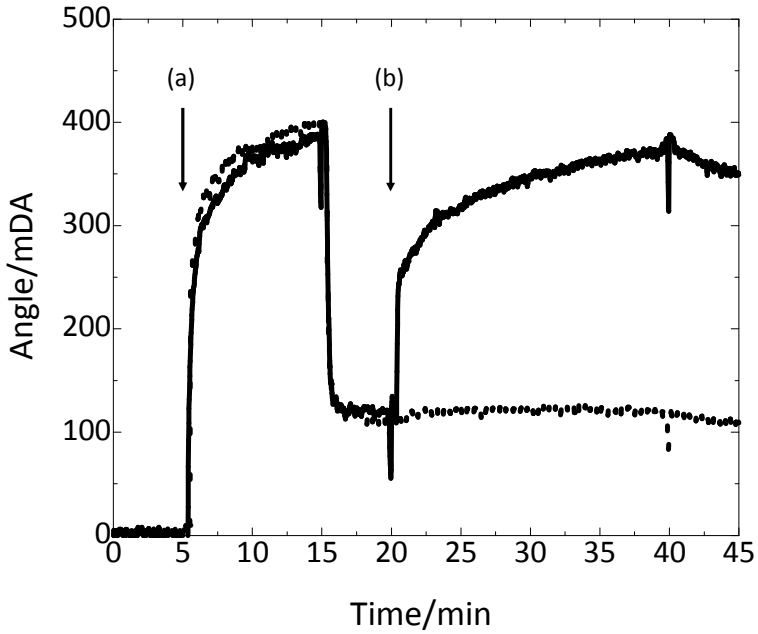


Figure S1. Surface plasmon resonance sensorigrams of interaction between oligo$(dA)_{20}$-UK and the oligo$(dT)_{20}$-incorporated surface. After a BSA solution was applied to block non-specific sites on the oligo$(dT)_{20}$-incorporated substrate (a), oligo$(dA)_{20}$-UK (solid line) or oligo$(dT)_{20}$-UK (dotted line) was allowed to flow (b).

Figure S1 shows the binding of oligo$(dA)_{20}$-UK onto the oligo$(dT)_{20}$ substrate, whereas no such increase was observed when oligo$(dT)_{20}$-UK was applied to the oligo$(dT)_{20}$ substrate, as shown by the dashed line in Figure S1. These results show that UK could be specifically conjugated on the surface through hybridization between oligo$(dA)_{20}$ on UK and oligo$(dT)_{20}$ on the surface. In addition, oligo$(dA)_{20}$-UK can be conjugated onto a cell surface modified with a oligo$(dT)_{20}$–PEG-lipid conjugate through DNA hybridization.

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Chapter 2

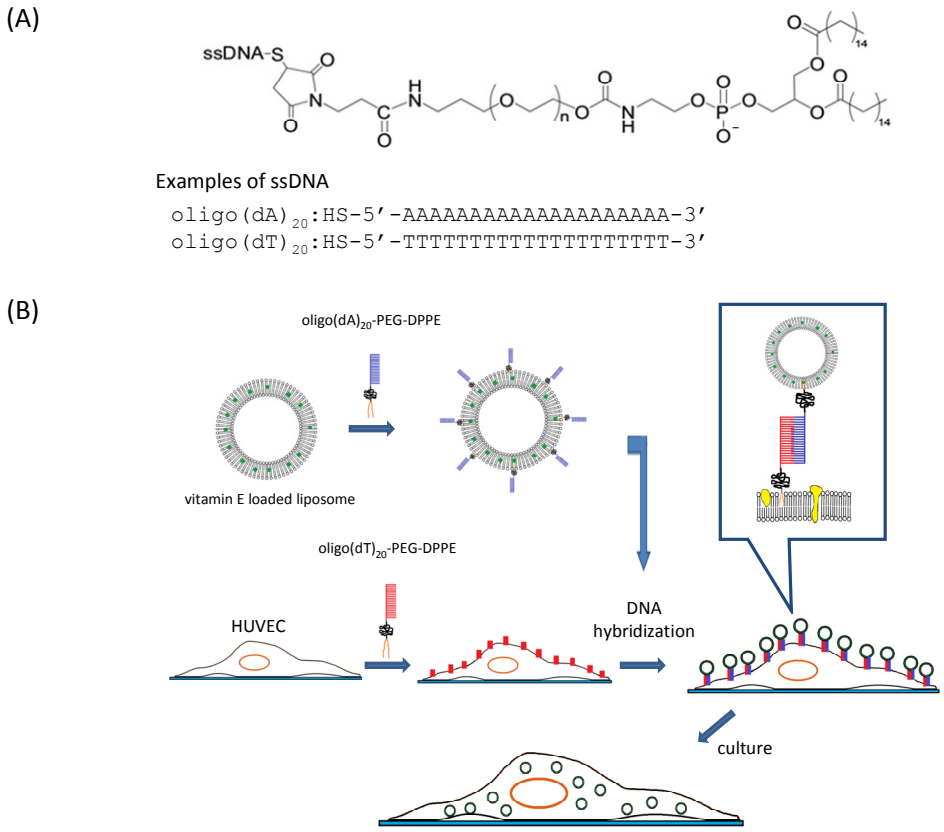
Introduction of Antioxidant-Loaded Liposomes into Endothelial Cell Surfaces through DNA Hybridization

INTRODUCTION

In organ transplantation, donated organs are stored in an organ preservation solution, such as EuroCollins solution or University of Wisconsin (UW) solution, without sufficient oxygen for a period of time. During cold ischemic storage of organs before transplantation, ischemia results in impairment of the antioxidant defense systems in cells; while, subsequent reperfusion of blood results in an increased concentration of reactive oxygen species (ROSs)[1] in the cells, especially endothelial cells, by blood-mediated reactions. ROSs appear to mediate tissue injury through lipid peroxidation and the activation of endothelial cells, resulting in damage to cell functions and structures.[2,3] A reduction of the ROSs produced in endothelial cells at the time of reperfusion suppresses the ischemia-reperfusion damage. Methods which can effectively deliver antioxidants to endothelial cells are desirable.

Recently, it has been demonstrated that cell surfaces can be modified through the immobilization of bioactive molecules, liposomes and other cells through covalent bond formation[4,5], electrostatic interaction[6,7], and hydrophobic interaction[8–10]. The function of membrane proteins likely deteriorate via the covalent bond method due to their

chemical modification. Cationic polymers and cationic liposomes are known to be cytotoxic. The hydrophobic interaction method, in which amphiphilic polymers such as PEG-lipids are used, is more versatile than other two. PEG-lipids rapidly anchor onto the cell membrane free of cytotoxic effects. In this chapter, this technique was examined for delivery of antioxidants to endothelial cells and suppression of ROSs production. Liposomes loaded with the antioxidant, vitamin E[11–13] (liposome-vitamin E[14,15]), were immobilized on the surface of human umbilical vein endothelial cells (HUVECs) using



Scheme 1. Immobilization of liposomes onto the surface of human umbilical vein endothelial cells (HUVECs). (A): Chemical structure of a ssDNA-PEG-lipid conjugate. (B): Schematic illustration of immobilization of liposomes onto HUVEC surfaces using ssDNA-PEG-lipids. Cells and liposomes were treated with oligo(dT)$_{20}$- and oligo(dA)$_{20}$-PEG-lipid, respectively. The lipid parts of the conjugates anchor onto the lipid bilayer of the cell membrane and the liposome through hydrophobic interactions. The liposome is then immobilized on the HUVEC surface through hybridization between oligo(dA)$_{20}$ on the liposomes and oligo(dT)$_{20}$ on the HUVECs.

amphiphilic poly(ethylene glycol)-phospholipid conjugates (PEG-lipids) carrying single-stranded oligonucleotides (ssDNA), as shown in Scheme 1. The liposomes and HUVECs were modified with PEG-lipids carrying 20-mers of deoxyadenylic acid (oligo$(dA)_{20}$) and 20-mers of complementary deoxythymidylic acid (oligo$(dT)_{20}$), respectively. It was expected that the liposomes would be immobilized on HUVECs through DNA hybridization between oligo$(dA)_{20}$ and oligo$(dT)_{20}$. The internalization of the liposomes into the cytosol of HUVECs and the antioxidant effect of vitamin E-loaded liposomes were examined *in vitro*.

EXPERIMENTAL PROCEDURES

Materials.

The following were purchased from the indicated suppliers: *α*-N-Hydroxysuccinimidyl-*ω*-maleimidyl poly(ethylene glycol) (NHS-PEG-Mal, MW 5000) and 1, 2-dipalmitoyl-*sn*-glycerol-3-phosphatidylethanolamine (DPPE) from NOF Corporation (Tokyo, Japan); L-*α*-phosphatidylcholine Type XVI-E from egg yolk (EggPC), bovine serum albumin (BSA), antimycin A from Streptomyces sp., and oligo$(dA)_{20}$ and oligo$(dT)_{20}$, which carry a protected SH group at the 5'-end (oligo$(dA)_{20}$-SH, oligo$(dT)_{20}$-SH) from Sigma-Aldrich Chemical Co. (St. Louis, MO); methanol, chloroform, dichloromethane, triethylamine, diethyl ether, calcium chloride dehydrate, and magnesium chloride hexahydrate from Nacalai Tesuque (Kyoto, Japan); Hoechst 33342 for nuclear stain from Dojindo Laboratories (Kumamoto, Japan); 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), Fluorescent LDL complex

Alexa594, and SYBR® Green II from Invitrogen Co. (Carlsbad, CA); phosphate-buffered saline (PBS) from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan); the Phospholipids C-test Kit, DL-α-tocopherol (vitamin E), and dimethyl sulfoxide from Wako Pure Chemical (Osaka, Japan); Sephadex G-25 M and NAP-5 columns from GE Healthcare UK Ltd., (Buckinghamshire, UK); 1-Palmitoyl-2-[12-[(7-nitro-2-1, 3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphoethanolamine (NBD-lipid) from Avanti Polar Lipids, Inc. (Alabaster, Alabama); human umbilical vein endothelial cell (HUVECs), cell culture medium EBM-2, EGM™-2-MV SingleQuots™, trypsin 0.025 %/EDTA 0.01 % in HEPES and trypsin neutralizing solution from Lonza Walkersville, Inc. (Walkersville, MD); and nitrocellulose membranes (0.8 μm pore size) and Millex-GP 0.22 μm filter units from Millipore Co. (Billerica, MA).

Synthesis of Oligo(dA)$_{20}$- or Oligo(dT)$_{20}$- Conjugated PEG-DPPE.

Oligo(dA)$_{20}$- or oligo(dT)$_{20}$-conjugated PEG-DPPE (oligo(dA)$_{20}$–PEG-DPPE or oligo(dT)$_{20}$–PEG-DPPE) (Scheme 1(A)) were synthesized following the method reported previously.[9,10] Briefly, DPPE (25 mg), NHS-PEG(5000)-Mal (200 mg), triethylamine (three drops) and dichloromethane (5 mL) were mixed and stirred for 72 hours at room temperature. The mixture was poured into ice chilled diethyl ether to precipitate Mal-PEG-DPPE. After suction filtration, Mal-PEG-DPPE was dissolved into benzene and then freeze dried.

A protected SH group was removed from oligo(dA)$_{20}$ and oligo(dT)$_{20}$ according to the manufacturer's instructions and oligo(dA)$_{20}$-SH and oligo(dT)$_{20}$-SH were obtained. Mal-PEG-DPPE and oligo(dA)$_{20}$-SH or oligo(dT)$_{20}$-SH were dissolved in

PBS and stirred overnight to obtain oligo$(dA)_{20}$–PED-DPPE or oligo$(dT)_{20}$–PEG-DPPE. To confirm coupling between Mal-PEG-DPPE and oligo$(dA)_{20}$-SH or oligo$(dT)_{20}$-SH, the reaction mixtures were analyzed using 10% polyacrylamide gel electrophoresis. ssDNA in the gel was stained with SYBR® Green II, and observed by ImageQuant LAS 4000 (GE Healthcare UK Ltd., Buckinghamshire, UK). The reaction products were used without purification.

Preparation of Liposomes Surface Modified with Oligo$(dA)_{20}$ and Oligo$(dT)_{20}$.

EggPC (20 mg) and varying amounts of vitamin E (0.5 mg, 1.0 mg, 2.0 mg or 4.0 mg) were dissolved in 4.0 mL of chloroform; a dry thin film formed on the inner surface of a round-bottom flask by drying the solution under reduced pressure using a rotary evaporator. The film was hydrated with 2 mL of PBS solution and then vigorously stirred overnight at room temperature to prepare the suspension of lipid vesicles. The suspension was extruded through a series of membrane filters with different pore sizes: 0.8 μm (once), 0.22 μm (twice), and 0.1 μm (ten times). Small and uniform liposomes carrying vitamin E were obtained.

The diameter of the liposomes was determined by a dynamic light scattering spectrometer (DLS-7000; Otsuka Electronics Co., Ltd, Osaka, Japan). The resultant liposome suspensions were applied to Sephadex G-25 M columns to separate the liposomes carrying vitamin E (liposome-vitamin E) from free vitamin E. Fractions (1.5 mL) were collected. The liposomes were dissolved by adding methanol to determine vitamin E contents. The concentrations of vitamin E and lipids in the methanol solutions were determined by UV-VIS absorption at 284 nm (DU 640; Beckman Coulter Inc., Brea, California) and with the Phospholipids C-test Kit, respectively.

Twenty milligram of EggPC and 0.2 mg of fluorescence-labeled lipid (NBD-lipid) were dissolved in 2 mL of chloroform. Small and uniform liposomes carrying 0.9 mol% liposome-NBD were prepared as described above. Oligo$(dA)_{20}$ and oligo$(dT)_{20}$ were introduced to the liposome surface by adding 200 μL of oligo$(dA)_{20}$–PEG-DPPE or oligo$(dT)_{20}$–PEG-DPPE (0.5 mg/mL in PBS) to 1.0 mL of the liposome suspension (5.0 mg/mL in PBS) and incubating for 2 hours at room temperature.

Surface Plasmon Resonance Analyses of the Interaction of the Oligo$(dA)_{20}$−PEG-Liposomes and Oligo$(dT)_{20}$−PEG-Liposomes Using a Gold Surface Carrying Oligo$(dT)_{20}$.

An in-house–designed surface plasmon resonance (SPR) instrument[16] was used to follow immobilization of liposomes through DNA hybridization. The SPR angle shifts were monitored at 37 °C. Gold-coated BK-7 glass plates (refractive index: 1.515, size: 25 × 25 × 1 mm, Arteglass Associates Co., Kyoto, Japan) were washed thoroughly with pure water and 2-propanol sequentially and then dried under nitrogen gas. A glass plate was set in the SPR flow cell. Oligo$(dT)_{20}$ was immobilized on the gold surface by applying a solution of oligo$(dT)_{20}$-SH (10 μg/mL in PBS) into the SPR cell. A BSA solution (10 mg/mL in PBS) was applied to reduce nonspecific adsorption of oligo$(dA)_{20}$–PEG-liposomes on the sensor surface. A solution of oligo$(dA)_{20}$–PEG-liposome-vitamin E (25 μg/mL in PBS) was applied to the surface at a flow rate of 4.0 mL/min. In a control experiment, a solution of oligo$(dT)_{20}$–PEG-liposome-vitamin E (25 μg/mL in PBS) was also applied to see the effect of the nucleotide sequence.

Fluorescence Recovery of NBD-Liposome − Immobilized Gold Surface after Photobleaching.

Gold-coated BK-7 glass plates were washed thoroughly with pure water and 2-propanol sequentially and then dried under nitrogen gas. Oligo$(dT)_{20}$ was immobilized on the gold surface by applying a solution of oligo$(dT)_{20}$-SH (50 μg/mL in PBS) for 20 minutes at room temperature. A BSA solution (10 mg/mL in PBS) was applied to reduce nonspecific adsorption of oligo$(dA)_{20}$–PEG-liposomes-NBD on the surface for 10 minutes at room temperature. A solution of oligo$(dA)_{20}$–PEG-liposome-NBD (100 μg/mL in PBS) was applied to the surface for 1 hour at room temperature. A small spot (diameter: 40 μm) on the liposome-NBD–immobilized gold surface was photobleached and observed over time under a fluorescence microscope (ECLIPSE Ti-E, Nikon Co., Tokyo, Japan).

Observation of Liposome Immobilization on the Surface of HUVECs by DNA Hybridization.

HUVECs (Figure S1) were cultured on a glass bottom dish in 2 mL of EBM-2 supplemented with EGM™-2-MV SingleQuots™ at 37 °C under 5% CO_2. Oligo$(dT)_{20}$ was introduced to the cell surface by adding a solution of 500 μL of oligo$(dT)_{20}$–PEG-DPPE solution (0.5 mg/mL in PBS) to the culture medium and the cells were cultured for 1 hour at 37 °C. The cell surface was washed three times with PBS containing 0.9 mM calcium chloride and 0.33 mM magnesium chloride (PBS(+)) to remove oligo$(dT)_{20}$-SH and oligo$(dT)_{20}$-S-S-oligo$(dT)_{20}$ contaminating the oligo$(dT)_{20}$–PEG-DPPE solution. Then, 500 μL of a oligo$(dA)_{20}$–PEG-liposome-NBD solution (0.5 mg/mL in PBS) was added to the culture medium and the cells were left

for 30 minutes at room temperature. The cell surface was washed three times with PBS(+). The cells were observed over time under a confocal laser scanning microscope (FluoView, FV10i, Olympus Optical Co. Ltd., Tokyo, Japan). Untreated cells or cells treated with oligo$(dT)_{20}$–PEG-liposome-NBD were also observed.

Protection of HUVECs from Oxidative Stress by Treatment with Liposome-Vitamin E.

Liposomes with/without vitamin E and a free vitamin E solution were examined to study their protective effects from oxidative stress. HUVECs were cultured on 6.0 cm φ dishes in 5 mL of EBM-2 supplemented with EGM™-2-MV SingleQuots™. Cells were modified with oligo$(dT)_{20}$–PEG-DPPE and washed with PBS(+) to remove oligo$(dT)_{20}$-SH and oligo$(dT)_{20}$-S-S-oligo$(dT)_{20}$. The modified cells were incubated with oligo$(dT)_{20}$–PEG-liposome or oligo$(dA)_{20}$–PEG-liposome with/without vitamin E for 30 minutes. Cells were treated with free vitamin E solution to test the effect of liposomes for 30 minutes. After a certain period of culture, two mL of 100 μM antimycin A solution, which was prepared by adding 1 mL of antimycin A solution (2.5 mg antimycin A in 1 mL DMSO) to 99 mL of EBM-2, was added to the culture medium as a source of oxidative stress to the cells. After 1 hour incubation, the medium was replaced with that containing 10 μM H_2DCFDA. After 30 minutes incubation, the cells were analyzed by a fluorescence-activated cell analyzer (Guava EasyCyte Mini; Millipore, Billerica, MA, USA) equipped with a 488 nm diode laser; 50,000 cells were analyzed and histograms were generated. Untreated cells treated with and without antimycin A were used as positive and negative controls, respectively.

RESULTS

Characterization of ssDNA-PEG-DPPEs and Vitamin E-Loaded Liposomes.

ssDNA–PEG-DPPEs (Scheme 1(A)) were synthesized according to the method reported previously.[9,10] Coupling reactions between Mal-PEG-DPPE and

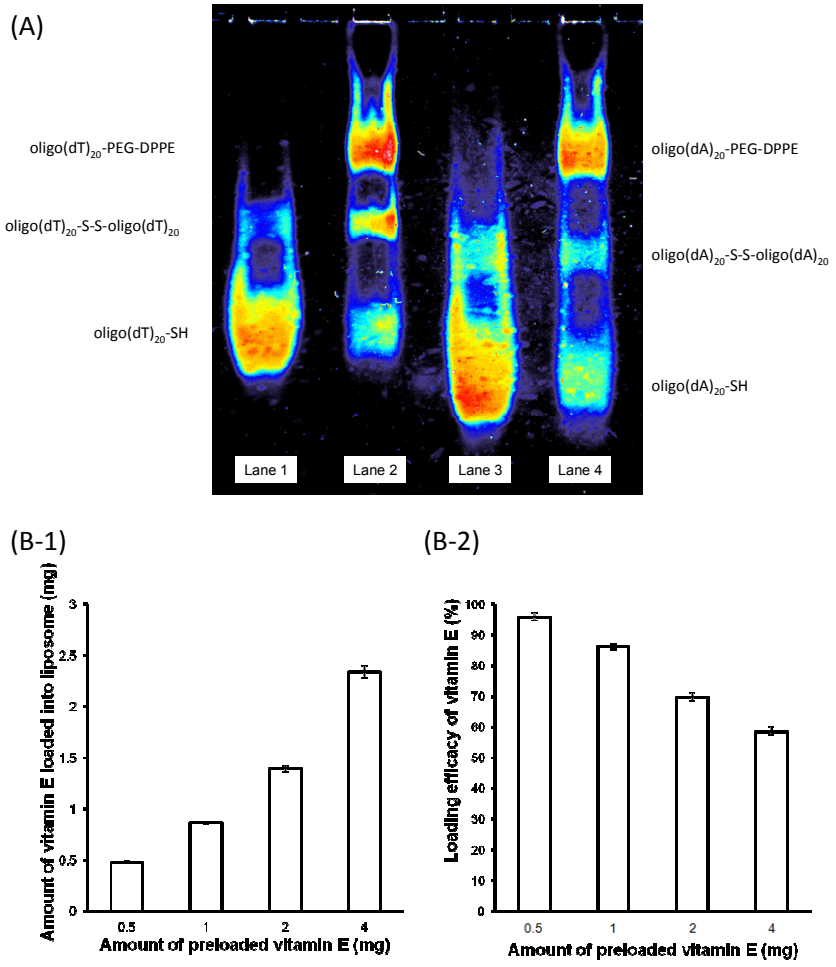


Figure 1. Characterization of a Mal-PEG-DPPE/ssDNA-SH reaction mixture and vitamin E-loaded liposomes. (A): Polyacrylamide gel electrophoresis. Lane 1: oligo(dT)$_{20}$-SH, Lane 2: Reaction mixture of Mal-PEG-DPPE and oligo(dT)$_{20}$-SH, Lane 3: oligo(dA)$_{20}$-SH. Lane 4: Reaction mixture of Mal-PEG-DPPE and oligo(dA)$_{20}$-SH. (B): Vitamin E loaded into liposomes. (B-1): The amount of vitamin E in the liposome compared with the amount mixed with EggPC during preparation. (B-2): Loading efficiency of vitamin E for varying amounts mixed with EggPC.

oligo$(dA)_{20}$-SH or oligo$(dT)_{20}$-SH were done. The reaction mixtures were analyzed by electrophoresis using a 10% polyacrylamide gel as shown Figure 1(A). Three bands were observed: ssDNA–PEG-DPPE, ssDNA-S-S-ssDNA and ssDNA-SH from the upper to the lower band, respectively. These results indicate that the coupling reaction was successful, although residual unreacted ssDNA and side reaction products, ssDNA-S-S-ssDNA were observed.

A lipid film with a vitamin E coating on the inner surface of a round-bottom flask was hydrated and suspended in PBS with vigorous agitation, and size of the liposomes were adjusted by extruding the lipid suspension through a series of membrane filters. The average diameter of the vitamin E-loaded liposome, determined by dynamic light scattering, was 118 ± 12 nm. After the liposomes were modified with oligo$(dA)_{20}$–PEG-DPPE, their average diameter was 119 ± 9 nm. Figure 1(B) shows the loading amounts and efficiency of vitamin E into liposomes. Liposomes containing 0.07 mg vitamin E/mg EggPC were used in the following experiments, that is, about 90 mol% lipid and 10 mol% vitamin E in the liposome.

Liposome Immobilization on the Cell Surface through DNA Hybridization.

Scheme 1 shows the method for immobilizing liposomes on the cell surface through DNA hybridization. When the oligo$(dT)_{20}$–PEG-DPPE is added to a cell suspension, the hydrophobic lipid part, DPPE, spontaneously anchors into the lipid bilayer of the cell membrane through hydrophobic interactions.[9,10] The hydrophilic oligo$(dT)_{20}$-PEG segments should remain on the cell surface. Oligo$(dT)_{20}$ were used for immobilization of liposomes carrying oligo$(dA)_{20}$ on the cell surface through the hybridization of oligo$(dA)_{20}$ and oligo$(dT)_{20}$.

The SPR method, which serves to monitor events on a gold surface, was employed to examine the binding and stability of oligo(dA)$_{20}$-modified liposomes immobilized through DNA hybridization with oligo(dT)$_{20}$ on the gold thin layer in real time. Figure

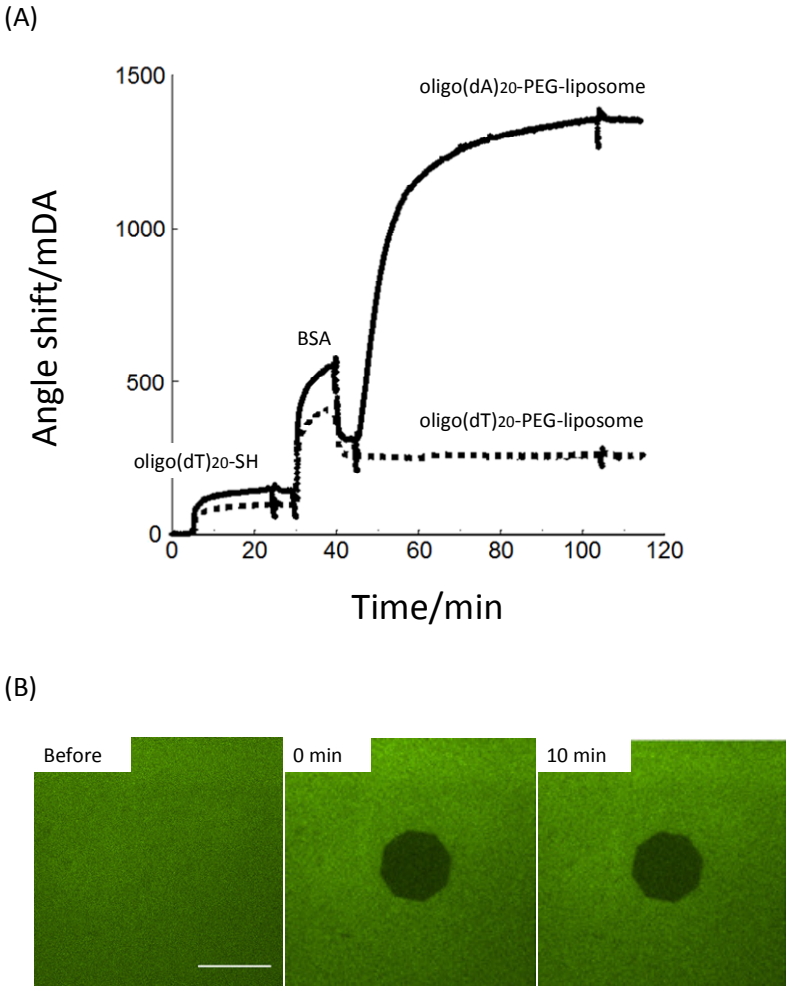


Figure 2. (A) Surface plasmon resonance sensorgrams of the interaction between oligo(dA)$_{20}$-liposomes and the oligo(dT)$_{20}$ immobilized surface. A solution of oligo(dT)$_{20}$-SH was perfused onto a flow cell to immobilize oligo(dT)$_{20}$ on a SPR sensor surface through thiol/gold bonding. A BSA solution was applied to block non-specific sites on the oligo(dT)$_{20}$-incorporated substrate. Then, a suspension of oligo(dA)$_{20}$–PEG-liposomes (solid line) or oligo(dT)$_{20}$–PEG-liposomes (dashed line) was introduced to the oligo(dT)$_{20}$ immobilized SPR sensor. DA: degrees of angle. (B) Liposome-NBD coverage and fluorescence recovery of a photobleached spot on the liposome-immobilized gold surface by DNA hybridization. Scale bar: 40 μm.

2(A) shows the SPR profiles for interactions between oligo$(dA)_{20}$–PEG-liposomes or oligo$(dT)_{20}$–PEG-liposomes and oligo$(dT)_{20}$ immobilized on the SPR sensor surface. The initial increases in the SPR profiles show immobilization of oligo$(dT)_{20}$-SH on the gold surface through the Au/thiol reaction. The second increase of the SPR angle was due to exposure of the sensor surface to a BSA solution to block non-specific immobilization of oligo$(dA)_{20}$ or oligo$(dT)_{20}$–PEG-liposomes. The solid line shows the SPR sensorgram when the sensor surface carrying oligo$(dT)_{20}$ was exposed to a oligo$(dA)_{20}$–PEG-liposome suspension; while, the dashed line shows when the sensor surface carrying oligo$(dT)_{20}$ was exposed to a oligo$(dT)_{20}$–PEG-liposome suspension. The third increase of the sensorgram, indicated by the solid line, demonstrated the immobilization of oligo$(dA)_{20}$–PEG-liposomes onto the oligo$(dT)_{20}$ substrate through DNA hybridization. No such increase was observed when a solution of oligo$(dT)_{20}$–PEG-liposome was applied to the oligo$(dT)_{20}$ substrate, as shown by the dashed line in Figure 2(A). When the SPR sensor surface was washed with PBS after the oligo$(dA)_{20}$–PEG-liposomes were immobilized, no practical SPR angle decrease was observed. These results indicate that the oligo$(dA)_{20}$–PEG-liposomes could be specifically immobilized on the SPR sensor surface through DNA hybridization and remained stable.

The immobilization of liposomes on gold surfaces through DNA hybridization was also investigated with fluorescence microscopy. The surface was uniformly stained green as shown in Figure 2(B). Figure 2(B) also includes an image of photobleaching experiments. Fluorescence did not recover at the photobleached spot during a 10 minutes observation period. It has been established that the fluorescence intensity of two dimensional lipid bilayers on substrates containing fluorescent probes recover

within 10 minutes when photobleached.[17] These results indicate that liposomes were uniformly immobilized on the substrate without deteriorating into a two dimensional lipid bilayer.

Immobilization of Liposomes on HUVEC Surfaces.

Liposomes were immobilized on the surface of HUVECs and their stability during culture was examined. Liposomes carrying oligo(dA)$_{20}$ and the fluorescent dye, NBD, that is oligo(dA)$_{20}$–PEG-liposome-NBD, were employed to trace liposomes after application to HUVECs. A solution of oligo(dT)$_{20}$–PEG-DPPE was added to HUVECs. After free oligo(dT)$_{20}$–PEG-DPPE was removed by washing the cells with PBS(+), a

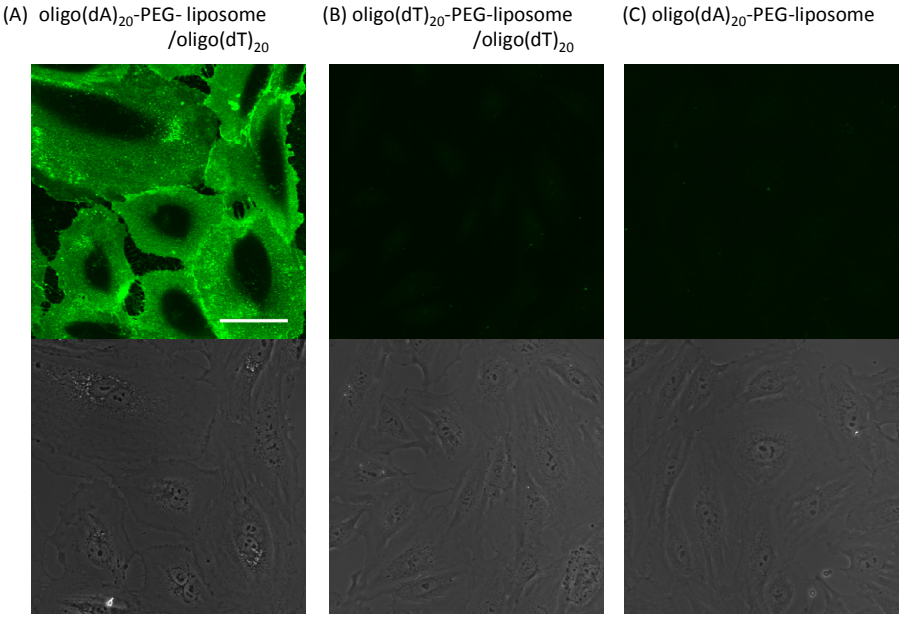


Figure 3. Immobilization of liposome-NBD onto HUVECs through DNA hybridization. (A): HUVECs were modified with oligo(dT)$_{20}$-PEG-DPPE and then treated with complementary oligo(dA)$_{20}$–PEG-liposome-NBD. (B): HUVECs were modified with oligo(dT)$_{20}$–PEG-DPPE and then, treated with the mismatched oligo(dT)$_{20}$–PEG-liposome-NBD. (C): HUVECs without modification with oligo(dT)$_{20}$–PEG-DPPE were treated with oligo(dA)$_{20}$–PEG-liposome-NBD. Upper: confocal fluorescent microscopic images. Lower: phase contrast microscopic images. Scale bar: 50 μm.

suspension of oligo$(dA)_{20}$–PEG-liposome-NBD was applied to the cell and left for 30 minutes at 37 °C. The cells were observed under a confocal laser scanning microscope after washing with PBS(+). Fluorescence from NBD could be clearly and homogenously observed on the cell membrane of each cell, but no fluorescence was observed where the nucleus is located, as shown in Figure 3(A). The cell was thicker where the nucleus was located; thus, the nucleus was not observable by the confocal laser scanning microscope. By contrast, no fluorescence was observed on the surface of the cells treated with oligo$(dT)_{20}$–PEG-liposome-NBD (Figure 3(B)) or the cells not pretreated with oligo$(dT)_{20}$–PEG-DPPE and treated with oligo$(dA)_{20}$–PEG-liposome-NBD (Figure 3(C)). These results clearly demonstrate that liposomes-NBD could be immobilized on HUVECs through hybridization between oligo$(dT)_{20}$ and oligo$(dA)_{20}$, as shown in Scheme 1. Liposome-NBD–immobilized HUVECs were cultured for different time periods and observed by a confocal laser scanning microscope. Although the fluorescence was observed uniformly on the cell membrane just after treatment with liposome-NBD, fluorescent particles were observed over the course of 24 hours of observation, as shown in Figure 4(A). In addition, the vertical fluorescence images, which were reconstructed from the horizontal images, clearly showed that liposome-NBD immobilized on the HUVEC surface moved to the inside of the cells (Figure 4(B)). After liposome-NBD immobilization, the cells were cultured for varying times and analyzed by a fluorescence-activated cell analyzer. Control cells were not treated with liposome-NBD. Although the NBD fluorescence intensity of the cells decreased, it remained at a high level even after 12 hours (Figure 4(C)). The decrease in fluorescenced intensity was mainly due to cell division during culture.

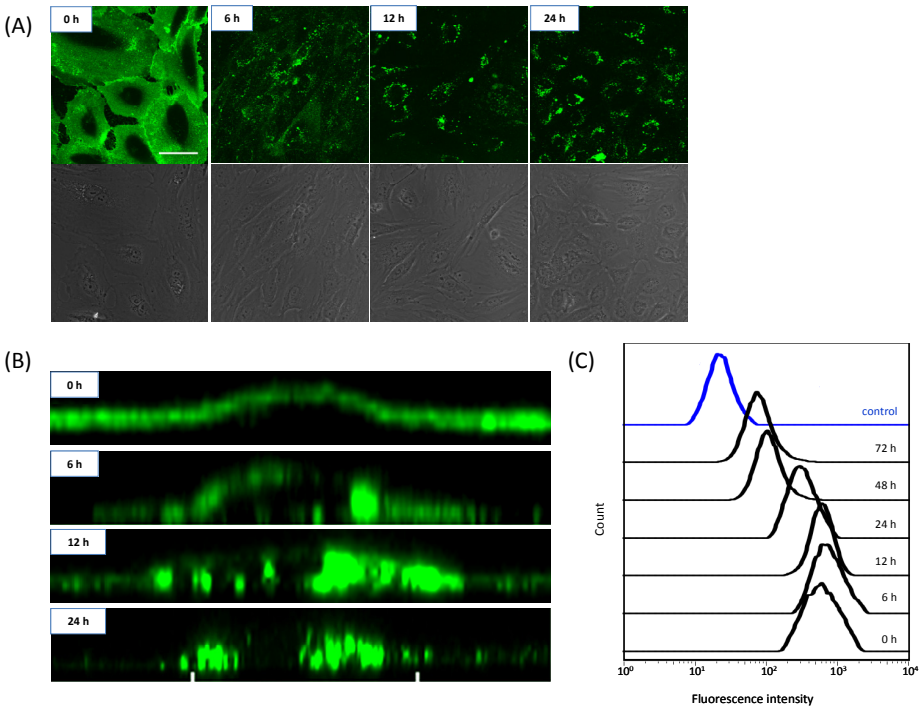


Figure 4. Fate of liposome-NBD immobilized on HUVECs. Liposome-NBD was immobilized on the HUVEC surface by DNA hybridization. The cells were cultured in medium at 37 °C and observed at predetermined periods of time. (A): Upper pictures: confocal laser scanning fluorescence microscopic images. Lower pictures: phase contrast microscopic images. Scale bar: 50 μm. (B): Perpendicular fluorescence microscopic images constructed from fluorescence intensities of horizontal images in at a certain section. (C): Fluorescence-activated cell analyses of HUVECs immobilized with liposome-NBD by DNA hybridization.

Protection of HUVECs from Oxidative Stress by Treatment with Liposome-Vitamin E.

HUVECs were pretreated with liposomes with/without vitamin E or free vitamin E. After a certain period of time, antimycin A, which blocks the mitochondrial electron transport system,[18] was added to the culture medium to exert oxidative stress on the cells. The amounts of ROS produced in the cells were monitored using H_2DCFDA, which reacts with ROS in cells yielding fluorescence.[19,20] Results of

fluorescence-activated cell analyses of the cells are shown in Figure 5. The naïve cell stressed by antimycin A produced strong florescence, as shown by red lines in Figure 5 (A, B, C, D), indicating ROS production in the cells. The fluorescence intensities of the cells treated with liposome-vitamin E carrying complementary ssDNA decreased. ROS production was most effectively inhibited when antimycin A was added 12 hours after the cells were treated with liposome-vitamin E as shown in Figure 5 (A). On the contrary, the fluorescence intensities of the cells treated with liposome-vitamin E carrying mismatched ssDNA or liposomes without vitamin E carrying complementary

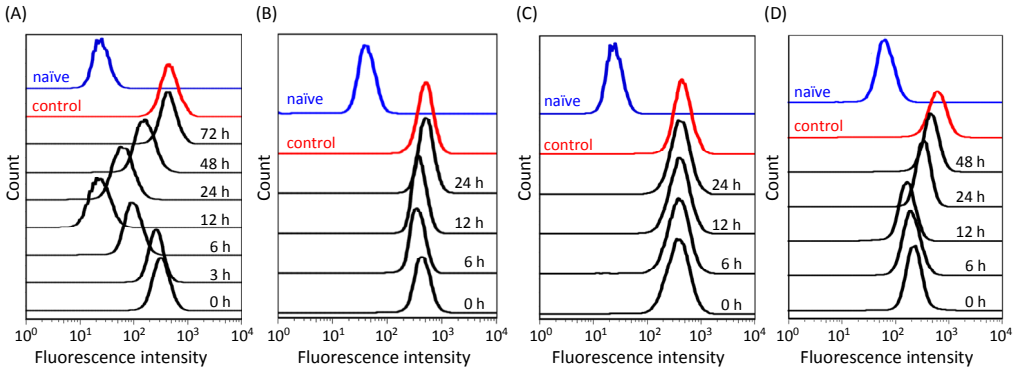


Figure 5. Antioxidant activity of liposome-vitamin E in HUVECs exposed to antimycin A. (A): HUVECs were modified with oligo(dT)$_{20}$–PEG-DPPE and then treated with oligo(dA)$_{20}$–PEG-liposome-vitamin E. (B): HUVECs were modified with oligo(dT)$_{20}$–PEG-DPPE and then treated with oligo(dT)$_{20}$–PEG-liposome-vitamin E. (C): HUVECs were modified with oligo(dT)$_{20}$–PEG-DPPE and then treated with oligo(dA)$_{20}$–PEG-liposome without vitamin E. (D): HUVECs were cultured in medium supplemented with vitamin E. These cells were washed with PBS(+) to remove free liposomes with/without vitamin E and free vitamin E after 30 min culture. Antimycin A was applied to culture medium at predetermined times after HUVECs were treated with liposomes with/without vitamin E or free vitamin E solution. When HUVECs are exposed to antimycin A, ROSs are produced. The ROSs convert H_2DCFDA to species with strong fluorescence. HUVECs were analyzed by a fluorescence-activated cell analyzer to determine the amounts of ROSs produced in the cell. Naïve HUVECs were used as a negative control (blue line), and those exposed to antimycin A were used as a positive control (red line).

ssDNA increased strongly as was the case with the naïve cells, as shown Figure 5 (B, C). When free vitamin E in DMSO was added to the culture medium at 100 μg/ mL, vitamin E concentration was higher than that of liposome-vitamin E solution (vitamin E concentration; 35 μg/mL), the fluorescence intensities of the cells decreased perceptibly but approximately only a tenth of that of the liposome-vitamin E carrying complementary ssDNA (Figure 5 (A) and (D)).

DISCUSSION

In organ transplantation, it is important to suppress ischemia-reperfusion damage induced by intracellular ROSs, which are produced by blood-mediated reactions at the time of reperfusion. As shown in Figure 3, liposomes carrying vitamin E could be immobilized on HUVECs using amphiphilic ssDNA–PEG-lipids. The immobilized-liposomes carrying vitamin E were internalized into HUVECs (Figure 4(B)). Production of ROSs was effectively inhibited 6 - 48 hours after immobilization of liposome-vitamin E on the HUVECs (Figure 5(A)). The liposome-vitamin E could protect the cells from oxidative stress. In this chapter, the protective effect of free vitamin E was also examined. When a solution of free vitamin E (dissolved in 1% DMSO) was added to culture medium, it inhibited ROSs production in HUVECs 6 - 48 hours after treatment, though its effect was limited (Figure 5(D)). It is possible that hydrophobic vitamin E can adsorb to the lipid cell membrane where it is then gradually internalized into the cells by the same process as liposome-vitamin E internalization. An organic solvent (DMSO), which is not suitable for use in donated organs, must be used

to deliver vitamin E to cells due to its insolubility in aqueous solutions. Although free vitamin E has some protective effect, the liposome-vitamin E carrying complementary ssDNA is superior to free vitamin E.

Iwata and colleagues previously found liposomes carrying argatroban, a low molecular weight anticoagulant drug, were immobilized on islets of Langerhans using ssDNA–PEG-lipids by the method shown in Scheme 1.[9] Transplantation of islets of Langerhans (islets) is an effective treatment for type 1 diabetes patients and has been performed clinically for more than 15 years. Unfortunately, more than 50% of islets are destroyed immediately after intraportal transplantation.[21, 22] Islets that are exposed to the blood stream induce blood coagulation. Early graft loss is caused by the instant blood mediated inflammatory response (IBMIR) followed by blood coagulation.[22-25] Iwata and colleagues examined immobilization of liposomes carrying argatroban on islets of Langerhans (islets) for inhibition of IBMIR. They found that liposomes on islets were gradually released from the islet surface to culture medium; thus, antithrombin activity in the media increased with time. Here, it was found that liposomes carrying vitamin E were gradually internalized into HUVECs after several hours of culture, as shown in Figure 4(B). Huth *et al.* reported that HUVECs internalized liposomes via clathrin-dependent endocytosis, caveolae-dependent endocytosis and macropinocytosis, and that other cell types internalized by means of different mechanism.[26] It was expected that the fate of liposomes immobilized on cells depends highly on the kinds of cells. Liposome-NBD was immobilized on fibroblasts and smooth muscle cells, and its fate was examined (Figure S2). Fluorescence intensity decreased during 24 h culture, and a small amount of liposome-NBD was internalized while some also remained near the cell membrane.

The results obtained in this chapter suggest that liposome-vitamin E effectively protects the endothelial cells of a donated organ from reperfusion damage, although further studies, especially *ex vivo* and *in vivo* studies, are necessary to determine whether this method is clinically useful for organ transplantation. The employment of ssDNA–PEG-lipids has many potential applications in biomedical research and also clinical cell therapies.

SUPPORTING INFORMATION

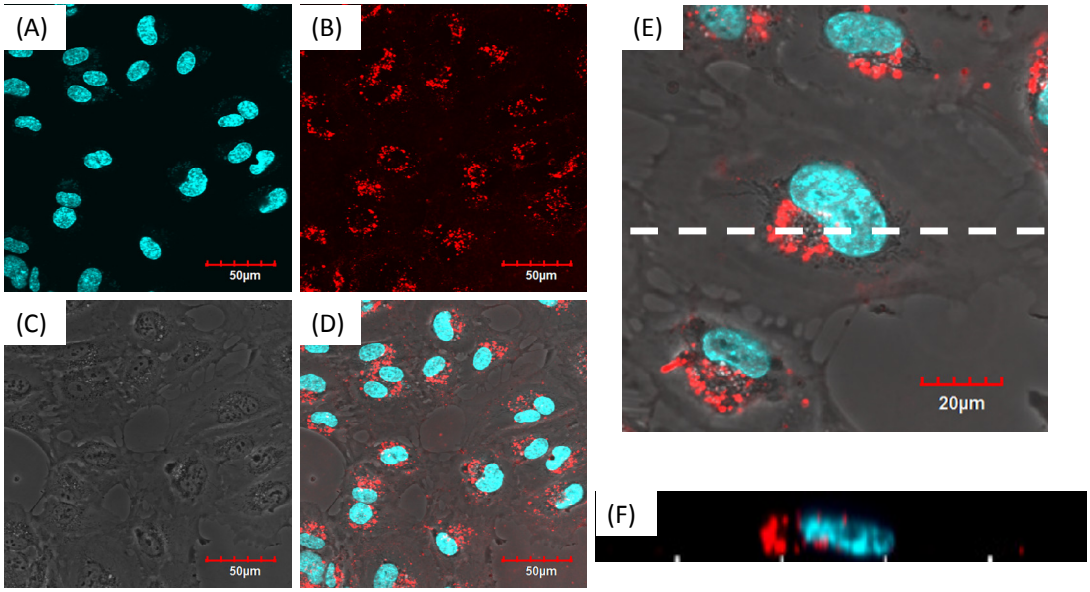


Figure S1. Confirmation of cells as HUVECs using Fluorescent LDL complex Alexa594. After washing cells on a glass bottom dish with PBS, a solution of Fluorescent LDL complex Alexa594 (red, 5.0 μg/mL in EBM-2) was added and the cells were incubated for 1 hour. Then, Hoechst solution (blue, 0.5 μg/mL in EBM-2) was added and the cells incubated for an additional 15 minutes. After washing with PBS, the cells were observed under a confocal laser scanning microscope: (A) Fluorescent LDL complex Alexa594, (B) Hoechst, (C) phase contrast microscopic image, (D) Merged images of Fluorescent LDL complex Alexa594 and Hoechst, (E) Magnified image and (F) A cross sectional image of a cell, which was constructed from the fluorescence intensities of horizontal images along the white dashed line in (E).

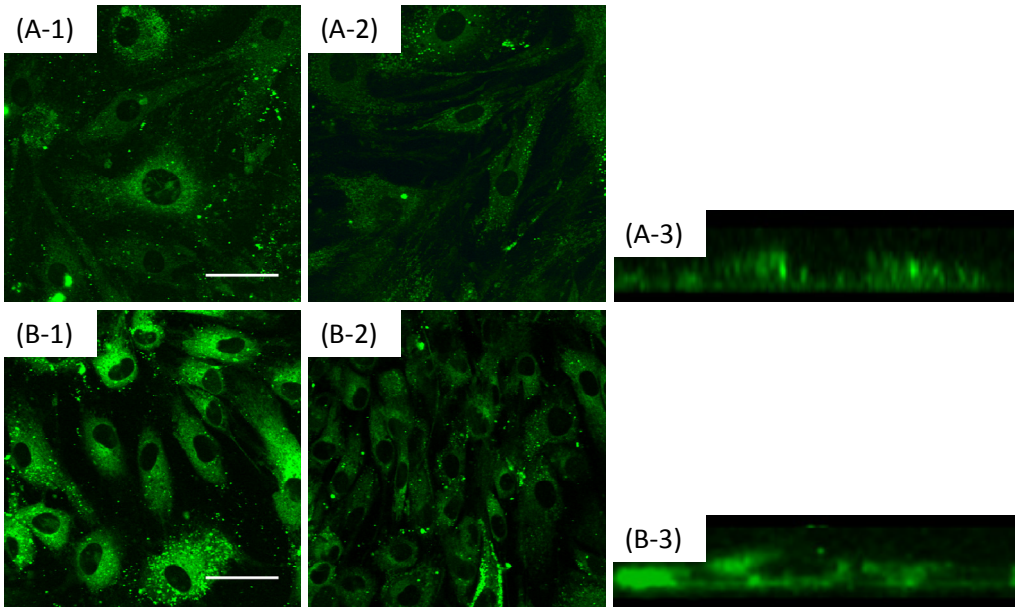


Figure S2. Confocal fluorescence microscopic images of fibroblasts and smooth muscle cells treated with liposome-NBD. Liposome-NBD was immobilized on the cell surface by DNA hybridization. (A-1) and (A-2): Images of fibroblasts just after immobilization of liposome-NBD and after culture for 24 hours, respectively. Scale bar: 50 μm. (A-3): Perpendicular fluorescence microscopic image of fibroblasts after culture for 24 hours constructed from fluorescence intensities of horizontal images in at a certain section. (B-1) and (B-2): Images of smooth muscle cells just after immobilization of liposome-NBD and after culture for 24 hours, respectively. Scale bar: 50 μm. (B-3): Perpendicular fluorescence microscopic image of smooth muscle cells after culture for 24 hours constructed from fluorescence intensities of horizontal images in at a certain section. In the case of HUVECs, uniform fluorescence was observed on the cell membrane just after treatment with liposome-NBD, but fluorescent particles were observed over the course of 24 hours, as shown in Figure 4(A). No such fluorescent particles, however, were observed in either cell after 24 hours culture, although these two kinds of cells took up less liposome-NBD.

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Chapter 3

Immobilization of Sertoli Cells on Islets of Langerhans

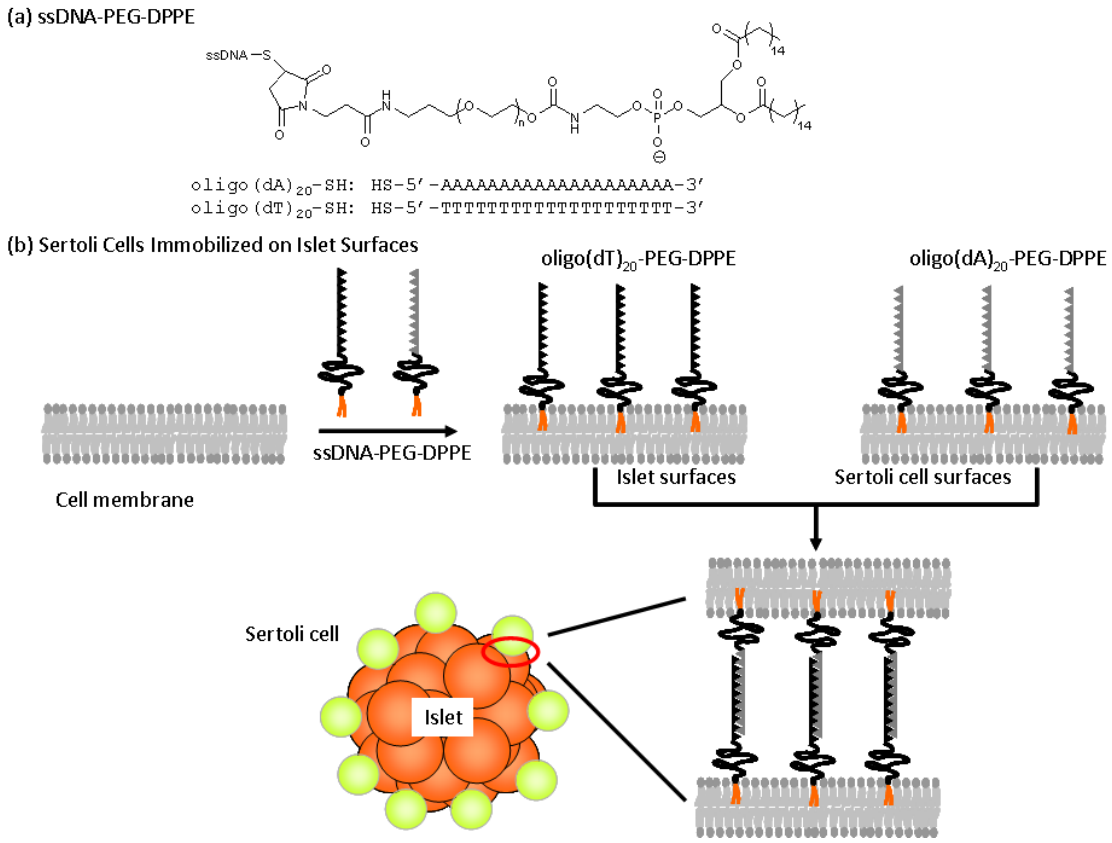
INTRODUCTION

In clinical islet transplantation, immunosuppressive therapy is required to control rejection reactions to the graft by the host immune system. Although steroid-free immunosuppressive therapy has been introduced in current islet transplantations,[1] there are still risks of some side effects, such as opportunistic infections, malignant tumors and impairment of islet functions.[2] Alternatives to administrating immunosuppressive drugs are needed to overcome these problems. One approach is the bioartificial pancreas, in which the islets are enclosed within a semi-permeable membrane.[3,4] Other approaches, such as ultra-violet light radiation of islets,[5] culture of islets under low temperature,[6] and co-transplantation of islets with Sertoli cells,[7,8] have been tested.

Sertoli cells play a crucial role in creating the immunoprivileged environment of the testis[9] by secreting immunoprotective factors[10,11] and forming a barrier to inhibit infiltrating lymphocytes.[10,12,13] Some groups reported long term survival of islets when co-transplanted with Sertoli cells[7,8] into the subcapsular site of the kidney for co-localization. However, the co-localization of Sertoli cells with islets is difficult in the liver because for clinical islet transplantation islets and Sertoli cells are to be transfused into the portal vein together. The islets congregate in larger veins in the liver, but Sertoli

cells are usually carried to more peripheral smaller veins by the blood flow.

It has been reported that conjugates of single-stranded oligonucleotides (ssDNA) and long alkyl chains can be used to attach cells to other cells without deterioration of cell survival and functions.[14–18] In previous studies, Iwata and colleagues synthesized conjugates of ssDNA and poly(ethylene glycol)-conjugated phospholipid (ssDNA–PEG-lipid) and employed them to immobilize cells on an islet[19] for improvement of graft survival.



Scheme 1. Immobilization of Sertoli cells onto an islet using ssDNA-PEG-DPPEs. (a) Oligo(dA)$_{20}$ and oligo(dT)$_{20}$ were used as ssDNA conjugated to PEG-DPPE. (b) A schematic illustration of the anchoring of ssDNA-PEG-DPPE to a lipid bilayer of the cell membrane and immobilization of Sertoli cells onto an islet through hybridization between oligo(dA)$_{20}$ and oligo(dT)$_{20}$.

In this chapter, ssDNA–PEG-lipid was applied to immobilize Sertoli cells on the islet surface, as shown in Scheme 1. The surface of Sertoli cells and islets were modified with 20-mers of deoxyadenylic acid (oligo$(dA)_{20}$) and 20-mers of complementary deoxythymidylic acid (oligo$(dT)_{20}$), respectively, conjugated to PEG-lipids. Sertoli cells were immobilized on islets through DNA hybridization between oligo$(dA)_{20}$ and oligo$(dT)_{20}$. The cell composites of Sertoli cells and islets were infused into the mouse portal vein to examine the co-localization of Sertoli cells with islets.

EXPERIMENTAL PROCEDURES

Materials.

The following were purchased from the indicated suppliers: α-N-hydroxysuccinimidyl-ω-maleimidyl poly(ethylene glycol) (NHS-PEG-Mal, MW 5000) and 1,2-dipalmitoyl-*sn*-glycerol-3-phosphatidylethanolamine (DPPE) from NOF Corporation (Tokyo, Japan); oligo$(dA)_{20}$ and oligo$(dT)_{20}$, which carry a protected SH group at the 5'-end (oligo$(dA)_{20}$-SH, oligo$(dT)_{20}$-SH) or fluorescein isothiocyanate (FITC) at the 5'-end (FITC-oligo$(dA)_{20}$, FITC-oligo$(dT)_{20}$) from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA); dichloromethane, triethylamine, chloroform, diethyl ether, Blocking One, trypsin, penicillin–streptomycin mixed solution and heparin sodium salt from Nacalai Tesuque (Kyoto, Japan); ethylenediamine tetraacetic acid (EDTA) and Hoechst 33258 nuclear stain from Dojindo Laboratories (Kumamoto, Japan); Alexa 488-labeled rabbit anti-goat IgG, DNase,

N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer solution, Hanks' balanced salt solution (HBSS), and Medium 199 from Invitrogen Co. (Carlsbad, CA, USA); fetal bovine serum (FBS) from Equitech-Bio, Inc. (Kerrville, TX, USA); phosphate-buffered saline (PBS) from Nissui Pharmaceutical Co. Ltd (Tokyo, Japan); enzyme-linked immunosorbent assay (ELISA) kits for the insulin assay from Shibayagi Co., Ltd (Gunma, Japan) and for Activin A from R&D Systems, Inc. (Minneapolis, MN, USA); polyclonal goat antimouse GATA-4 from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); polyclonal guinea pig anti-swine insulin from Dako (Glostrup, Denmark); tetrarhodamine isothiocyanate (TRITC)-labeled rabbit anti-guinea pig IgG from Abcam plc. (Cambridge, UK); CellTracker™ Green CMFDA from Lonza Walkersville, Inc. (Walkersville, MD, USA); formalin solution, dithiothreitol (DTT), Tween 20, and Triton X-100 from Wako Pure Chemical (Osaka, Japan); Collagenase from Nitta Gelatin (Osaka, Japan); the NAP-5 column from GE healthcare (GE Healthcare UK Ltd, Buckinghamshire, UK); and Tissue-Tek® from Sakura Fine Technical Co., Ltd (Tokyo, Japan).

Isolation of Sertoli Cells and Islets.

All animal experiments were approved by the Kyoto University Animal Care Committee. Sertoli cells were isolated from BALB/c mice (7 day old males, Japan SLC, Inc., Shizuoka, Japan) by Korbutt's method.[7] Briefly, testis were collected from mice, chopped into paste with scissors, and digested for 10 minutes at 37 °C in a collagenase solution (2.5 mg/mL in HBSS). The digest was washed with HBSS supplemented with 1 mM EDTA (HBSS/EDTA) and 0.5% bovine serum albumin, additionally digested for 10 minutes at 37 °C with trypsin (25 μg/mL) and DNase (4 μg/mL) in HBSS/EDTA,

and washed with HBSS. The final cell pellet was resuspended in Medium 199 supplemented with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin. The cells were cultured in the medium for 3 days after isolation.

To identify Sertoli cells, the isolated cells were immunohistochemically stained for GATA-4, which is a transcription factor and known to be abundantly expressed in the nuclei of Sertoli cells.[20,21] Cells were fixed in 4% formalin solution at 37 °C for 15 minutes, permeabilized in 0.2% Triton X-100 in PBS at room temperature for 15 minutes, treated with Blocking One for 1 hour to block nonspecific binding of antibodies and then washed with PBS. The cells were immersed in 2% polyclonal goat anti-mouse GATA-4 in Blocking One and left for 1 hour at room temperature, followed by washing with PBS. The cells were incubated with fluorescent labeled secondary antibody, 0.2% Alexa 488-labeled rabbit anti-goat IgG in Blocking One at room temperature for 1 hour, followed by washing with PBS containing 0.05% Tween 20. Cell nuclei were counterstained with Hoechst 33258. The stained cells were observed under a fluorescence microscope (IX71, Olympus Optical, Co., Ltd, Tokyo, Japan). Population of GATA-4 positive cells was elucidated from 300 cells counts.

Islets were isolated from BALB/c mice (6 week old males, Japan SLC, Inc., Shizuoka, Japan) by the collagenase digestion method.[22] Briefly, the pancreas was collected from mice, chopped into paste with scissors, and digested for 18 minutes at 37 °C in a collagenase solution (0.5 mg/mL in HBSS). Islets were isolated from the digest by Ficoll density gradient purification, and cultured for 3 days in Medium 199 supplemented with 10% FBS, 8.8 mM HEPES buffer, 100 units/mL penicillin, 100 μg/mL streptomycin, and 8.8 units/mL heparin to remove cells damaged by the isolation procedure.

Immobilization of Sertoli Cells onto Islets.

Oligo$(dA)_{20}$– and oligo$(dT)_{20}$–PEG-DPPEs, which were synthesized from Mal-PEG-DPPE and oligo$(dA)_{20}$- or oligo$(dT)_{20}$-SH as described previously,[19] were used for surface modification of Sertoli cells and islets, respectively. A solution of oligo$(dT)_{20}$–PEG-DPPE (50 μL, 500 μg/mL in PBS) was added to an islet suspension in serum-free medium, and the mixture was incubated at room temperature for 1.5 hours. Oligo$(dT)_{20}$–PEG-DPPE modified islets were washed three times with serum-free medium and then mixed with a solution of FITC-oligo$(dA)_{20}$ (100 μL, 500 μg/mL in PBS), washed with serum-free medium and observed under a confocal laser scanning microscope (FLUOVIEW, FV500, Olympus Optical Co. Ltd, Tokyo, Japan) to examine the presence of oligo$(dT)_{20}$ on islets and the hybridization with oligo$(dA)_{20}$. The fluorophore was excited by an argon laser (488 nm) and the fluorescence was detected through a bandpass filter (510–550 nm).

Sertoli cell pellets (2×10^6 cells) were collected by centrifugation (180g, 5 min at 25 °C) after treatment with trypsin. The cells were washed with HBSS to remove the medium. After the addition of oligo$(dA)_{20}$–PEG-DPPE solution (100 μL, 500 μg/mL in PBS) to the cell pellet, the cell suspension was incubated for 30 minutes with gentle agitation at room temperature. After washing with HBSS, oligo$(dA)_{20}$–PEG-DPPE–modified cells were obtained by centrifugation ($180 \times g$, 5 min, 25 °C, twice). To examine the presence of oligo$(dA)_{20}$ on Sertoli cells, the cells were mixed with a solution of FITC-oligo$(dT)_{20}$ (100 μL, 500 μg/mL in PBS), washed with HBSS and observed under a confocal laser scanning microscope.

Oligo$(dA)_{20}$–PEG-DPPE–modified Sertoli cells (2×10^6) and oligo$(dT)_{20}$–PEG-DPPE–modified islets (200 islets) were mixed in serum-free medium

(200 μL). The mixture was incubated for 60 minutes with gentle agitation at room temperature. The Sertoli cell-immobilized islets were obtained after washing with culture medium. The Sertoli cell-immobilized islets were immunohistochemically stained for GATA-4 and were observed under a confocal laser scanning microscope. In addition, Sertoli cells stained by CellTracker™ were also employed for easy finding of Sertoli cells after their transplantation.

Insulin and Activin A Secreted from Sertoli Cell-Immobilized Islets.

Sertoli cell-immobilized islets and naïve islets (50 islets each) were mixed with glucose solutions in Krebs–Ringer's buffer (KRB) at concentrations of 0.1 g/dL, 0.3 g/dL and 0.1 g/dL glucose sequentially for intervals of 1 hour in each solution at 37 °C. The supernatants were collected after each 1 h incubation and the insulin concentrations were determined by ELISA according to the manufacturer's instructions. Sertoli cell-immobilized islets and naïve islets (50 islets each) were incubated in serum-free medium at 37 °C for 24 hours. The supernatants were collected and their Activin A concentrations were determined by ELISA according to the manufacturer's instructions.

Intraportal Transplantation of Sertoli Cell-Immobilized Islets into the Liver.

BALB/c mice (6 week old males, Japan SLC, Inc.) were used as recipients. Sertoli cells were stained by CellTracker™ prior to immobilization. Sertoli cell-immobilized islets (500 islets/100 μL) were transplanted into the liver through a portal vein of the mice. Mice were anesthetized during surgery by mask inhalation of isoflurane using a specialized instrument (400 Anesthesia Unit; Univentor, Malta); the isoflurane concentration was 4.5% to 5.0% for induction and 2.0% for maintenance with

an airflow rate of 200 mL/min. Mice were sacrificed immediately after transplantation of Sertoli cellimmobilized islets. Livers were retrieved and then immersed in 4% formalin solution for 2 days at room temperature. The formalin solution was removed and the livers were then embedded in Tissue-Tek® for freezing. The frozen specimens were sliced into 6-μm thick sections for immunohistochemical staining. The sections were observed under a fluorescence microscope (BX51, Olympus Optical Co., Ltd, Tokyo, Japan).

Sertoli cell-immobilized islets (500 islets/100 μL) without CellTracker™ staining were also used for transplantation. As a control experiment, 100 μL of a mixed suspension of Sertoli cells (5×10^6 cells) and islets (500 islets) was infused into the liver through a portal vein. Immediately after transplantation, mice were sacrificed and paraffin-embedded liver specimens were sliced into sections 4-μm thick. After deparaffinization, the sections were permeabilized with 0.2% Triton X-100 in PBS at room temperature for 15 minutes. The samples were then treated with Blocking One for 1 hour, followed by washing with PBS. The sections were treated with 1% polyclonal guinea pig anti-swine insulin and 2% polyclonal goat anti-mouse GATA-4 in Blocking One for 1 hour at room temperature. The sections were then incubated with fluorescent labeled secondary antibody, 2% TRITC-labeled rabbit antiguinea pig IgG and 0.2% Alexa 488-labeled rabbit anti-goat IgG in Blocking One, at room temperature for 1 hour followed by washing with PBS containing 0.05% Tween 20. The stained sections were observed under a fluorescence microscope.

RESULTS

Immobilization of Sertoli Cells onto Islets.

Amphiphilic polymers, ssDNA–PEG-DPPEs, were used to immobilize Sertoli cells onto islets through DNA hybridization between oligo$(dA)_{20}$ and oligo$(dT)_{20}$ as shown in Scheme 1. Sertoli cells were isolated from the testis of male BALB/c mice and seeded on a Petridish. Their purity was examined after 3 days culture by immunohistochemical staining for GATA-4, which is specifically expressed in the nuclei of Sertoli cells.[20,21] About 90% of cells were GATA-4 positive, as shown in Figure 1, and were used in the following experiments. Sertoli cells and islets were treated with oligo$(dA)_{20}$–PEG-DPPE and oligo$(dT)_{20}$–PEG-DPPE, respectively. Iwata and colleagues already reported that the surface modification with PEG-lipid did not

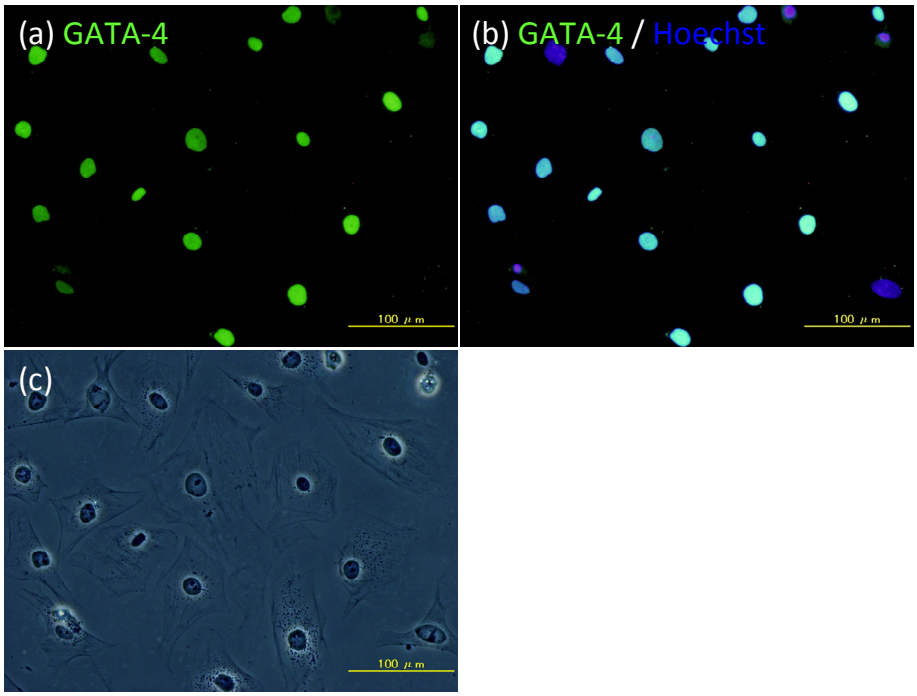


Figure 1. Immunohistochemical examination of Sertoli cells isolated from the testis of male BALB/c mice. (a) GATA-4 (green fluorescence), (b) GATA-4 (green fluorescence) and Hoechst 33258 (blue fluorescence), and (c) phase contrast microscopic image. Scale bar: 100 μm.

affect the islet function.[23] Viability of Sertoli cells after treatment with oligo(dA)$_{20}$–PEG-DPPE was 75.2 ± 4.2%, as estimated by the trypan blue exclusion method.

After washing with HBSS and serum-free medium, Sertoli cells and islets were treated with oligo(dA)$_{20}$–PEG-DPPE and oligo(dT)$_{20}$–PEG-DPPE, respectively. Each

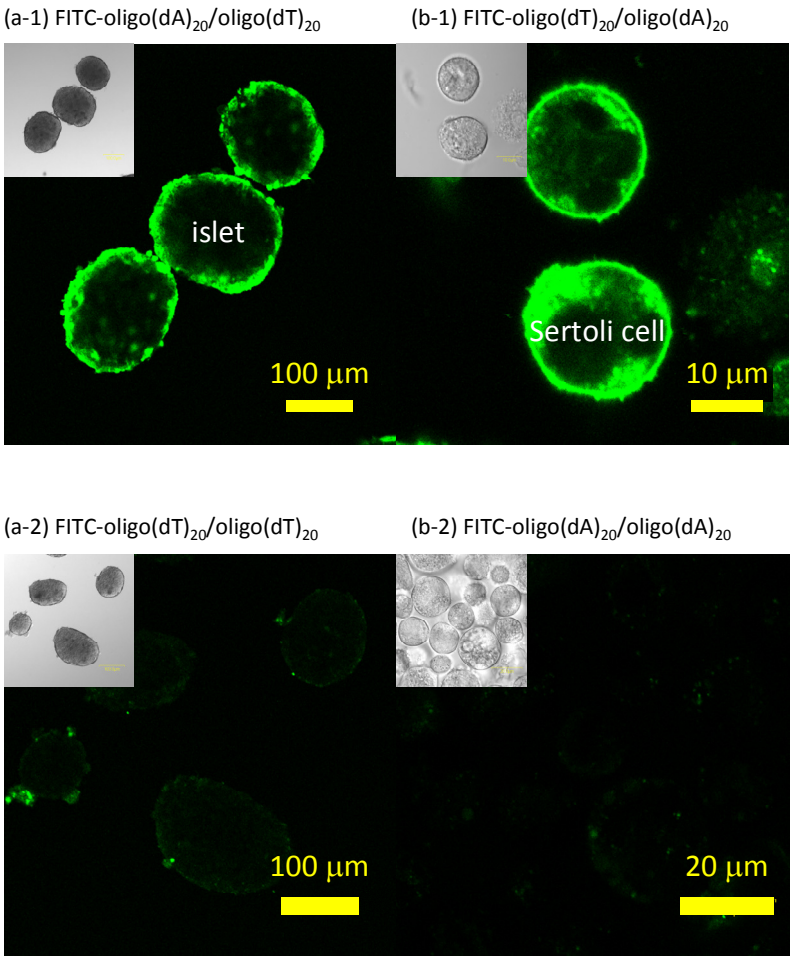


Figure 2. Surface modification of islets and Sertoli cells with ssDNA–PEG-DPPE. Sertoli cells and islets were observed under a confocal laser scanning fluorescent microscope. (a-1) FITC-oligo(dA)$_{20}$ fixed on islets modified with oligo(dT)$_{20}$–PEG-DPPE. (a-2) Islets were modified with oligo(dT)$_{20}$–PEG-DPPE and then mixed with FITC-oligo(dT)$_{20}$. (b-1) FITC-oligo(dT)$_{20}$ fixed on Sertoli cells modified with oligo(dA)$_{20}$–PEG-DPPE. (b-2) Sertoli cells were modified with oligo(dA)$_{20}$–PEG-DPPE and then FITC-oligo(dA)$_{20}$.

was then mixed with a solution containing the corresponding complementary FITC-ssDNA. They were then observed under a confocal laser microscope (Figure 2(a-1) and (b-1)). Bright fluorescent rings were found at the periphery of the islets and the Sertoli cells. When the Sertoli cells and islets were mixed with mismatched ssDNA, that is, FITC-oligo$(dA)_{20}$ and FITC-oligo$(dT)_{20}$ solutions, respectively, no fluorescent rings were seen (Figure 2(a-2) and (b-2)). These results indicate that FITC was immobilized on Sertoli cell and islet surface through hybridization between oligo$(dA)_{20}$ and oligo$(dT)_{20}$. Oligo$(dA)_{20}$– and oligo$(dT)_{20}$–PEG-DPPEs were present on Sertoli cells and islets, respectively, and maintained the ability to hybridize with complementary ssDNA, that is, FITC-oligo$(dT)_{20}$ and FITC-oligo$(dA)_{20}$.

Figure 3 shows that Sertoli cells were immobilized on islets through hybridization between oligo$(dA)_{20}$ and oligo$(dT)_{20}$, and contains images of the aggregates observed under a confocal florescence microscope. Sertoli cells and islets were treated with oligo$(dA)_{20}$–PEG-DPPE and oligo$(dT)_{20}$–PEG-DPPE, respectively. After washing with HBSS and serum-free medium, suspensions of each were mixed together and left for 60 minutes. Existence of Sertoli cells was examined by immunohistochemical staining of GATA-4 which was specifically expressed in Sertoli cells.[20,21] GATA-4 positive cells were found on the islet surface as shown in Figure 3(a). Sertoli cells stained with CellTracker™ were also used to see location of Sertoli cells in the aggregates. Sertoli cells were clearly observed at the periphery of the islets immediately after the immobilization (Figure 3(b)). However, few cells were found at the periphery of the islets when oligo$(dT)_{20}$–PEG-DPPE modified-islets were mixed with naïve Sertoli cells, that were observed under a phase contrast microscope (Figure 3(c)). When the aggregate prepared via hybridization were cultured for 4 days, some of Sertoli cells

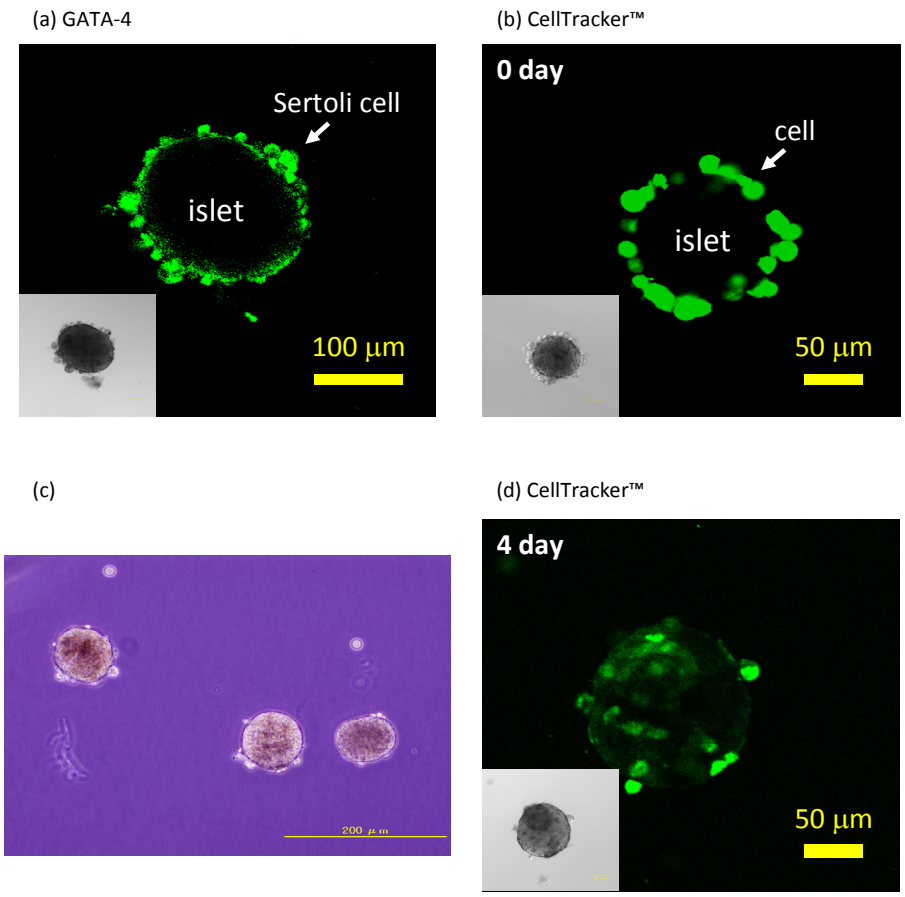


Figure 3. Immobilization of Sertoli cells onto islets. (a) Immunohistochemical staining of GATA-4 of Sertoli cells immobilized on an islet with the ssDNA–PEG-DPPE method. (b) Sertoli cells (stained with CellTracker™) immobilized on an islet with the ssDNA–PEG-DPPE method just after immobilization. (c) A phase contrast microscopic image of a mixture of oligo(dT)$_{20}$–PEG-DPPE-modified islets and naïve Sertoli cells. (d) Sertoli cells (stained with CellTracker™) immobilized on an islet after 4 days of culture. Insets: phase contrast microscopic image.

migrated into the inside of islets (Figure 3(d)). These results clearly demonstrate that Sertoli cells could be immobilized on islets through hybridization between oligo(dT)$_{20}$ on the islets and oligo(dA)$_{20}$ on the Sertoli cells.

In Vitro Functional Evaluation of the Sertoli Cell and Islet Composite.

Static glucose stimulation tests were performed on Sertoli cell-immobilized islets (50 islets) and islets without immobilization of Sertoli cells (naïve islets). As shown in

Figure 4(a), when the glucose concentration was increased from 0.1 to 0.3 g/dL, both naïve and Sertoli cell-immobilized islets increased insulin release. When the glucose concentration was again reduced to 0.1 g/dL, the insulin release returned to basal levels. No significant difference in insulin release was observed between the naïve and Sertoli cell-immobilized islets. These results indicate immobilization of Sertoli cells on the islet surface using ssDNA–PEG-DPPE did not impair the ability of islets to regulate insulin release.

Activin A is secreted by Sertoli cells[24] and was examined in Sertoli cells on islets to determine if they were active. Activin A was secreted from Sertoli cell-immobilized islets; whereas, no secretion was seen from naïve islets (Figure 4(b)). These results indicate immobilization of Sertoli cells on the islet surface using ssDNA–PEG-DPPE did not affect the ability of Sertoli cells to secrete Activin A. Sertoli cells could be immobilized onto islets without loss of function of islets or Sertoli cells.

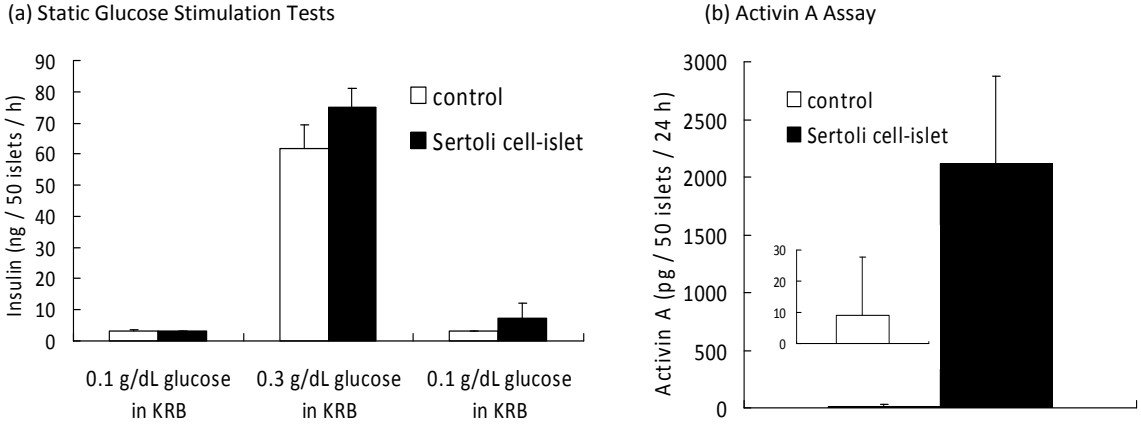


Figure 4. The functional evaluation of Sertoli cell-immobilized islets in vitro. (a) Glucose stimulation test of Sertoli cell-immobilized islets and naïve islets. Islets were sequentially exposed to 0.1, 0.3 and 0.1 g/dL glucose solutions in Krebs–Ringer buffer (KRB) at 37 °C for 1 hour each. The amounts of released insulin are expressed as mean ± SD for n = 3. (b) Activin A release from Sertoli cell-immobilized islets and naïve islets. The islets were incubated in serum-free medium at 37 °C for 24 hours. The amounts of released Activin A are expressed as mean ± SD for n = 5.

Co-localization of Sertoli Cells around Islets in the Liver.

Sertoli cell-immobilized islets were infused through the portal vein into the liver. Livers were retrieved from the recipient mice immediately after transplantation and thin sections prepared. When Sertoli cells were fluorescently stained with CellTracker™, fluorescently-stained cells were clearly observed at the periphery of the islet (Figure

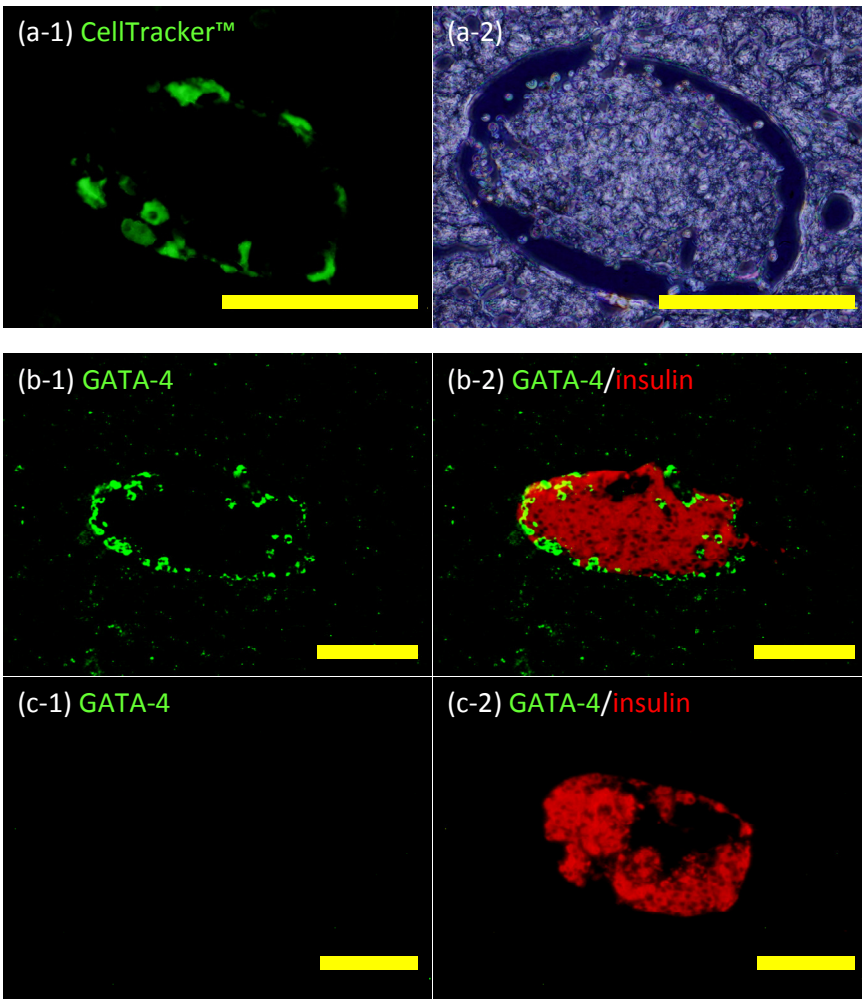


Figure 5. Histochemical analyses of Sertoli cells and islets in liver veins. (a) Sertoli cell-immobilized islets in the mouse liver: (a-1) CellTracker™ (green fluorescence) and (a-2) phase contrast microscopic image. (b) Sertoli cell-immobilized islets in the mouse liver: (b-1) GATA-4 (green fluorescence) and (b-2) Merged images of GATA-4 (green fluorescence) and insulin (red fluorescence). (c) Naïve islets in the mouse liver: (c-1) GATA-4 (green fluorescence) and (c-2) merged images of GATA-4 (green fluorescence) and insulin (red fluorescence). Scale bar: 100 μm.

5(a-1)). The cells were also immunohistochemically stained for GATA-4 to identify Sertoli cells not stained with CellTracker™. As seen in Figure 5(b), the islet was surrounded by GATA-4 positive cells. As a control, a simple mixture of Sertoli cells and islets was infused into the liver (Figure 5(c)). No GATA-4 positive cells could be found around these islets after the mixture was infused through the portal vein into the liver. These results clearly show ssDNA–PEG-DPPE is effective in localizing Sertoli cells on islets in the liver, even when they are exposed to blood flow.

DISCUSSION

Various bioactive substances, such as proteins,[25] liposomes[26] and cells,[19] have been immobilized on the cell surface using hetero-bifunctional polymers. One end of these polymers interacts with the cell membrane and another functional group is used to introduce a bioactive substance on the cell surface.[27,28] Several methods have been used to introduce polymers on the cell surface, including covalent conjugation of polymers carrying N-hydroxyl-succinimidyl esters to amino groups of membrane proteins,[27,28] electrostatic interactions between cationic polymers and the negatively charged cell surface,[27,28] and anchoring of long alkyl chains of amphiphilic polymers into the lipid bilayer of the cell membrane through hydrophobic interactions, as was done in this chapter. Each method has advantages and disadvantages. In the covalent conjugation methods, a stable bond forms between polymers and membrane proteins, but the functions of membrane proteins are often impaired.[29] The electrostatic interaction using cationic polymers simply involves adding a polymer solution to a cell suspension,

however, most cationic polymers are cytotoxic and most cells are deteriorated or killed after treatment with them.[30] In the hydrophobic interaction method, cells can be modified by simply adding amphiphilic polymers carrying long alkyl chains to a cell suspension and no major or clear deterioration of cell functions or integrity have been observed.[27,28] Thus, amphiphilic polymers were employed in this chapter.

In this method, bioactive substances are immobilized on the cell through the polymers. The avidin–biotin complex is most often used.[27,28] It can form stable bonding under physiological conditions and is suitable for *in vitro* examination of modified cells. Various avidins are commercially available, but are derived from albumen or bacteria. These avidins are hard to use for *in vivo* experiments, particularly in the clinical setting, because they are expected to induce unfavorable immune reactions. As an alternative, DNA duplex formation was employed. Oligo$(dA)_{20}$ and oligo$(dT)_{20}$ can form a stable complex under physiological conditions and the immunogenicity of DNA is much lower than that of avidin.

Sertoli cells could be immobilized on islets without loss of functions of either using amphiphilic ssDNA–PEG-DPPEs, as shown in Figure 4. The aggregates of Sertoli cells and islets could be co-transplanted into the mouse liver though the portal vein without detachment of Sertoli cells from the islet surface as shown in Figure 5. Further studies are necessary to determine if this method is clinically useful for the co-transplantation of Sertoli cells and islets in the liver. Factors to be examined include the stability of the cell composite, life span of Sertoli cells in the liver and the blood compatibility of Sertoli cells. However, cell immobilization using ssDNA–PEG-DPPEs is a versatile method to immobilize any kind of cells, such as regulatory T cells and endothelial cells, on islets without exerting harmful effects on the cells. It is expected

that this method will increase the success rate of islet transplantations.

Although the application of ssDNA–PEG-lipid to prolong graft survival of islets have been mainly focused on, this technology has many potential applications in modification of surface of cells and tissues with bioactive substances, study of cell–cell interaction and three dimensional tissue fabrication from dissociated single cells as discussed previously.[16,19]

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PART II

CO-AGGREGATION

Chapter 4

Transplantation of Co-aggregates of Sertoli Cells and Islet Cells into Liver Without Immunosuppression

INTRODUCTION

Transplantation of islets of Langerhans (islets) is a promising method for treating patients with insulin-dependent diabetes mellitus (type I diabetes). Worldwide, more than 200 clinical islet transplantations are carried out every year, and these transplantations require the recipients to go on long-term immunosuppressive therapy. The outcomes of islet transplantation have improved greatly in recent years because of the introduction of a steroid-free immunosuppressive protocol.[1] However, the side effects of immunosuppressive drugs, which included infection, malignant tumor formation, and impairment of islet functions, can themselves result in negative health effects.[2] Alternatives to administration of immunosuppressive drugs have been investigated, such as the use of a bioartificial pancreas[3] in which islets are enclosed within a semipermeable membrane,[4,5] radiation of islets with ultraviolet light,[6] culture of islets under lowtemperature conditions before transplantation,[7] and cotransplantation of islets and Sertoli cells.[8,9]

Sertoli cells play a crucial role in creating the immunoprivileged environment of the testis[10] by secreting immunoprotective factors[11–13] and by forming a barrier to

infiltrating lymphocytes.[11,14,15] Some groups have reported the long-termsurvival of islets that are co-transplanted with Sertoli cells.[8,9] In those studies, Sertoli cells and islets were transplanted into a renal subcapsular site in order to colocalize these two kinds of cells. However, co-localization of islets and Sertoli cells is challenging in clinical islet transplantation, as this technique mainly involves infusion of islets into the liver via the portal vein. The diameters of islets are much larger than the diameters of Sertoli cells, and islets are thus confined to larger veins in the liver; however, the smaller Sertoli cells can be carried by blood flow to the periphery of the liver via smaller veins.

In this chapter, co-aggregates of Sertoli cells and islet cells were prepared by the hanging drop culture method, and the aggregates were infused into liver through the portal vein. Co-localization of Sertoli cells and islet cells in the liver was examined using histochemical methods, and the protective effects of Sertoli cells on islet cells were examined in an allogeneic transplantation model using diabetic mice.

EXPERIMENTAL PROCEDURES

Materials.

The following reagents were purchased from the indicated suppliers: Blocking One, trypsin, penicillin-streptomycin mixed solution, and streptozotocin (STZ) from Nacalai Tesuque (Kyoto, Japan); ethylenediamine tetraacetic acid (EDTA) and Hoechst 33258 nuclear stain from Dojindo Laboratories (Kumamoto, Japan); Alexa 488-labeled rabbit anti-goat IgG, Alexa 488-labeled goat anti-guinea pig IgG, DNase, Hanks'

balanced salt solution (HBSS), Medium 199, and RPMI 1640 from Invitrogen Co. (Carlsbad, CA, USA); fetal bovine serum (FBS) from Equitech-Bio, Inc. (Kerrville, TX, USA); phosphate-buffered saline (PBS) from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan); enzyme-linked immunosorbent assay (ELISA) kits for the mouse insulin assay from Shibayagi Co., Ltd. (Gunma, Japan) and for Activin A from R&D Systems, Inc. (Minneapolis, MN, USA); polyclonal goat anti-mouse GATA-4 from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); polyclonal guinea pig anti-swine insulin from Dako (Glostrup, Denmark); tetrarhodamine isothiocyanate (TRITC)-labeled rabbit anti-guinea pig IgG from Abcam plc. (Cambridge, UK); CellTracker™ Orange CMRA from Lonza Walkersville, Inc. (Walkersville, MD, USA); formalin solution, tween 20, and Triton X-100 from Wako Pure Chemical (Osaka, Japan); collagenase from Nitta Gelatin (Osaka, Japan) and Tissue-Tek® from Sakura FineTechnical Co., Ltd. (Tokyo, Japan).

Preparation of Sertoli Cells and Islet Cells.

All animal experiments were approved by the Kyoto University Animal Care Committee. Sertoli cells were isolated from BALB/c mice (7-day-old male mice; Japan SLC, Inc., Shizuoka, Japan) by Korbutt's method.[8] Briefly, testes were collected from mice, chopped into paste with scissors, and digested for 10 minutes at 37°C in a collagenase solution (2.5 mg/mL in HBSS). The digested tissue was washed with HBSS supplemented with 1 mM EDTA (HBSS/EDTA) and 0.5 % bovine serum albumin, and cells were resuspended in a trypsin (25 μg/mL) and DNase (4 μg/mL) solution in HBSS/EDTA and digested further for 10 minutes at 37°C. Finally, the cells were washed with HBSS. The final cell pellet was resuspended in Medium 199 supplemented

with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. The cells were cultured in the medium for 3 days after isolation.

To identify Sertoli cells, the isolated cells were immunohistochemically stained for GATA-4, which is a transcription factor that is abundantly expressed in the nuclei of Sertoli cells.[16,17] The cells were fixed in 4% formalin solution at 37°C for 15 minutes, permeabilized by treatment with 0.2% Triton X-100 in PBS at room temperature for 15 minutes, treated with Blocking One for 1 hour to block nonspecific binding of antibodies, and then washed with PBS. The cells were immersed in 2% polyclonal goat anti-mouse GATA-4 in Blocking One and left for 1 hour at room temperature, followed by washing with PBS. The cells were incubated with fluorescently labeled secondary antibody (0.2% Alexa 488-labeled rabbit anti-goat IgG in Blocking One) at room temperature for 1 hour, followed by washing with PBS containing 0.05% Tween-20. Cell nuclei were counterstained with Hoechst 33258. The stained cells were observed under a fluorescence microscope (IX71, Olympus Optical, Co., Ltd., Tokyo, Japan). The proportion of GATA-4-positive cells was determined by counting 300 cells.

Islets were isolated from BALB/c or enhanced green fluorescent protein (EGFP) -transgenic C57BL/6 mice (6–8-week-old male mice; Japan SLC, Inc., Shizuoka, Japan) by the collagenase digestion method.[18] Briefly, a collagenase solution (0.5 mg/mL in HBSS) was injected into each mouse pancreas through the bile duct and then collected from the mouse and incubated for 18 minutes at 37°C for digestion. After the digested tissue had dissociated into pieces, the tissue was washed with HBSS and the islets were isolated from the digestion reaction using Ficoll density gradient purification. The isolated islets were cultured for 2 days in RPMI 1640 supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin to remove cells damaged by the

isolation procedure.

Islets were disintegrated into single cells by treatment with 1 mL of a trypsin solution (500 μg/mL in HBSS) at 37°C for 2 minutes then pipetted with a Pasteur pipette to apply mechanical force after addition of 1 mL medium. The single cells were resuspended in RPMI 1640 supplemented with 20% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. The islet cells were immunohistochemically stained for insulin to visualize β-cells, which are insulin-producing cells. The cells were fixed in 4% formalin solution at 37°C for 15 minutes, permeabilized with 0.2% Triton X-100 in PBS at room temperature for 15 minutes, treated with Blocking One for 1 hour to block nonspecific binding of antibodies, and washed with PBS. The cells were treated with 1% polyclonal guinea pig anti-swine insulin in Blocking One for 1 hour at room temperature and followed by washing with PBS. The cells were incubated with fluorescently labeled secondary antibody (2% TRITC-labeled rabbit anti-guinea pig IgG in Blocking One) at room temperature for 1 hour, followed by washing with PBS containing 0.05% Tween-20. Cell nuclei were counterstained with Hoechst 33258. The stained cells were observed under a fluorescence microscope (IX71, Olympus Optical, Co., Ltd., Tokyo, Japan).

Co-aggregation of Sertoli Cells and Islet Cells.

Sertoli cells were detached from culture dishes by treatment with a trypsin solution and collected by centrifugation (180 × *g*, 3 min at 25°C). The cells were then resuspended in RPMI 1640 supplemented with 20% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at densities of 1500 or 3000 cells/15 μL. Cells that were obtained by disintegration of islets were resuspended in RPMI 1640 supplemented with

20% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at densities of one half or 1 islet/15 μL. These suspensions of Sertoli cells and islet cells were mixed together and the cells were applied to a lid of a petri dish in 30-μL drops and cultured for hanging drop tissue culture for 4 days in a humidified incubator at 37°C in an atmosphere of 5% CO_2/95% air. To visualize the localization of Sertoli cells and islet cells in the aggregates, Sertoli cells were stained prior to visualization by CellTracker™ Orange according to the manufacturer's instructions, and islets isolated from EGFP-transgenic mice were used. The co-aggregates (each aggregate comprised 3000 Sertoli cells and islet cells from one islet) were observed under a confocal laser scanning microscope (FluoView, FV10i, Olympus Optical Co. Ltd., Tokyo, Japan).

Activin A and Insulin Secretion by Co-aggregates.

Fifty co-aggregates (each aggregate comprising 3000 Sertoli cells and islet cells from one islet) were incubated in serum-free RPMI 1640 at 37°C for 24 hours. The supernatants were collected, and the Activin A concentrations were determined by ELISA according to the manufacturer's instructions. Supernatants from cultures of 50 unmodified islets were used as a reference. The 50 co-aggregates were exposed sequentially to solutions of 0.1 g/dL, 0.3 g/dL, and 0.1 g/dL glucose in Krebs-Ringer's buffer (KRB) with incubation for 1 hour at 37°C for each solution. The supernatants were collected, and the insulin concentrations were determined by ELISA according to the manufacturer's instructions. The same glucose stimulation test was performed using 50 unmodified islets as a reference.

Intraportal Transplantation of Co-aggregates.

C57BL/6 mice (8 week old male mice; Japan SLC, Inc.) were used as recipients. C57BL/6 mice were induced diabetic by a single intraperitoneal injection of STZ (120 mg/kg body weight in citrate buffer, pH 4.2) 3–5 days before transplantation. Mice were used as diabetic recpients when their plasma glucose levels exceeded 450 mg/dL in two consecutive measurements. The diabetic mice were anesthetized during surgery by mask inhalation of isoflurane using a specialized instrument (400 Anesthesia Unit; Univentor, Malta); the isoflurane concentration was 4.5% to 5.0% for induction and 2.0% for maintenance with an airflow rate of 200 mL/min.

A suspension of 800 co-aggregates (each co-aggregate made of 1500 Sertoli cells and islet cells from 1/2 islet) were infused into the portal vein and transplanted in the liver. As a control experiment, unmodified islets (400 islets /100 μL) were infused through a portal vein. A total of 400 unmodified islets and or 800 co-aggregates that contained 1.2×10^6 Sertoli cells and islet cells from 400 islets were transplanted into each mouse to ensure that the number of islet cells was equal in the grafts. Plasma glucose levels were monitored every day during initial 30 days of the post-transplantation period, and were monitored every 2 days after that period. Normoglycemia was defined when two consecutive plasma glucose level measurements showed less than 300 mg/dL. Blood was collected from recipients and centrifuged to obtain plasma. Insulin level in the plasma was determined by ELISA according to the manufacturer's instructions. Recipients were rendered to the intraperitoneal glucose tolerance tests (IPGTTs) at 30 days post-transplantation to evaluate glucose tolerance.

Histochemical Analyses.

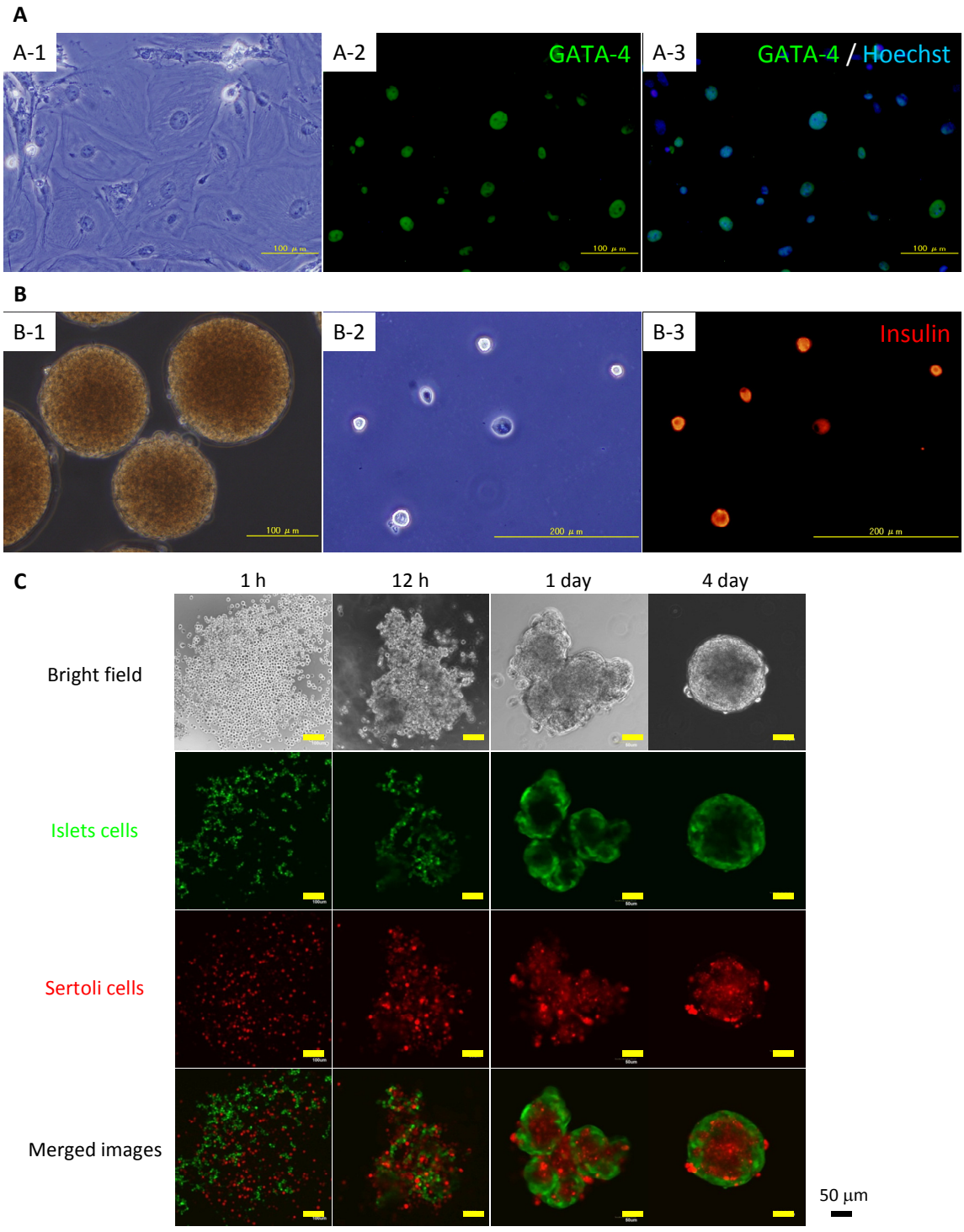
Some recipient mice were sacrificed at predetermined times after transplantation of the co-aggregates or unmodified islets. The livers were retrieved and immersed in 4% formalin solution for 2 days at room temperature. After the formalin solution was removed, the livers were dehydrated in alcohol solutions and embedded in paraffin. The livers were sliced into sections 4-μm in thickness. After deparaffinization, the sections were permeabilized with 0.2% Triton X-100 in PBS at room temperature for 15 minutes. The specimens were then treated with Blocking One for 1 hour followed by washing with PBS. Sections were treated with 1% polyclonal guinea pig anti-swine insulin in Blocking One for 1 hour at room temperature. The sections were then incubated with fluorescently labeled secondary antibody (0.2% Alexa 488-labeled goat anti-guinea pig IgG in Blocking One) at room temperature for 1 hour followed by washing with PBS containing 0.05% Tween-20. The stained specimens were observed under a fluorescence microscope (BX51, Olympus Optical Co., Ltd., Tokyo, Japan). The specimens were also stained with hematoxylin-eosin (HE) using a conventional staining protocol.

Figure 1. A, B, Immunohistochemical examination of Sertoli cells and islet cells. A, sertoli cells isolated from the testes of 7-day-old male BALB/c mice. (A-1) Phase contrast microscopic image of Sertoli cells cultured for 3 days. (A-2) Immunofluorescent staining of GATA-4 (green). (A-3) Merged image of Sertoli cells stained for GATA-4 (green) and Hoechst 33258 (blue). B, islets were isolated from the pancreata of 6- to 8-week-old male BALB/c mice. (B-1) Phase contrast microscopic image of islets cultured for 2 days. (B-2) Phase contrast microscopic image of islet cells. The islets were disintegrated by trypsin treatment. (B-3) Immunofluorescent staining of islet cells. C, segregation of Sertoli cells and islet cells in a co-aggregate during culture. A co-aggregate was prepared from Sertoli cells stained with CellTracker Orange and from islet cells isolated from EGFP-transgenic C57BL/6 mice by the hanging drop method. The cells were observed at the indicated times under a confocal laser scanning fluorescent microscope. Scale bar: 50 μm.

RESULTS

Preparations of Co-aggregates of Sertoli Cells and Islet Cells.

Sertoli cells were isolated fromthe testes of male BALB/c mice (Figure 1A, A-1).



Their purity was examined after 3 days of culture by immunohistochemical staining for GATA-4, which is a transcription factor that is abundantly expressed in the nuclei of Sertoli cells.[16,17] About 90% of the cells were GATA-4-positive, as shown in Figure 1A (A-2, A-3). Islets were isolated from male BALB/c mice (Figure 1B, B-1). The islets were disintegrated into single cells by treatment with trypsin (Figure 1B, B-2). Most of the cells were insulin-positive, as shown in Figure 1B (B-3),[19] and their viability after treatment with trypsin was greater than 93% as estimated by the trypan blue exclusion method. These Sertoli cells and islet cells were used in the following experiments.

A mixed suspension of Sertoli cells and islet cells were cultured for 4 days using the hanging drop culture method. Each 30-μL drop contained 3,000 Sertoli cells and islet cells from one islet. Figure 1C shows images of coaggregates comprising Sertoli cells stained with CellTracker Orange and islet cells isolated from EGFP-transgenic C57BL/6 mice. Although Sertoli cells and islet cells mixed randomly during the initial 12 hours of culture, during the following 12 hours of culture the cells segregated themselves such that Sertoli cells made up the core part of the co-aggregate while islet cells enclosed the Sertoli cells. These two cell types thus formed spherical aggregates, and cell segregation in the aggregates was even more apparent after 4 days of culture (Figure 1C).

In Vitro Functional Evaluation of the Co-aggregates.

Sertoli cells secrete Activin A.[20] The levels of Activin A in the culture medium were determined as a way to detect Sertoli cell activity in the co-aggregates. All aggregates showed high levels of Activin A secretion, whereas unmodified islets did not secrete Activin A (Figure 2A). These results indicate that Sertoli cells that were

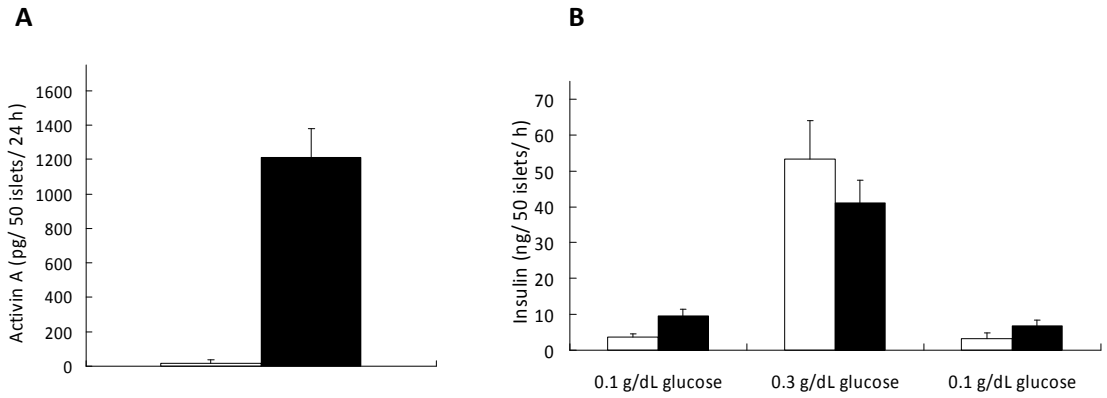


Figure 2. In vitro evaluation of Sertoli cells and islet cells in co-aggregates. A, the levels of Activin A released from unmodified islets and co-aggregates into serum-free media in a 24-hour period. The values are expressed as mean±SD for n=5 cultures. White bar on the left, islets; black bar, co-aggregates. B, glucose stimulates insulin release by unmodified islets and co-aggregates. Unmodified islets and co-aggregates were exposed sequentially for 1 hour to 0.1 g/dL, 0.3 g/dL, and 0.1 g/dL glucose in Krebs-Ringer buffer at 37°C. The values for the released insulin are expressed as mean±SD for n=3 cultures. White bar, islets; black bar, co-aggregates.

incorporated into the aggregate though the hanging drop culture method still maintained some of their original functions.

A glucose stimulation test was also performed on the co-aggregates to determine whether insulin secretion by the islet cells would change in response to changes in glucose levels. When the glucose concentration was increased from 0.1 g/dL to 0.3 g/dL, both the co-aggregates and unmodified islets showed increased levels of insulin release compared to basal levels (Figure 2B). When the glucose concentration was reduced to 0.1 g/dL after exposure to 0.3 g/dL glucose, insulin release returned to basal levels. There was no significant difference in the levels of insulin released in response to glucose between the co-aggregates and unmodified islets. These results indicate that the process of incorporating islet cells into co-aggregates through the hanging drop culture method did not impair their ability to regulate insulin release in response to changes in glucose levels.

Transplantation of Co-aggregates into the Livers of STZ-Induced Diabetic Mice.

Co-aggregates of cells from BALB/c mice or unmodified BALB/c islets were transplanted into the livers of diabetic C57BL/6 mice through the portal veins. The co-aggregates obstructed the blood vessels in the liver, indicating that smaller co-aggregates were needed to reduce damage to the liver. Thus, for transplantation, the

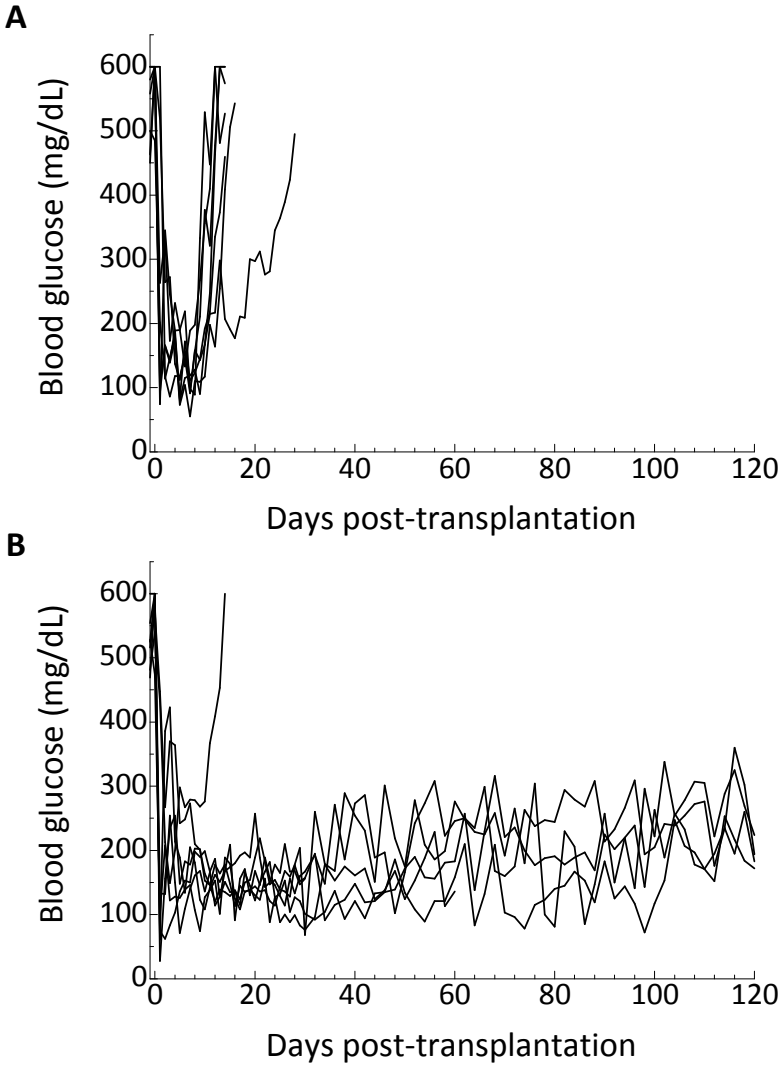


Figure 3. Non-fasting blood glucose levels of streptozotocin (STZ)-induced diabetic C57BL/6 mice after intraportal transplantation of (A) 400 unmodified islets (n=7mice) or (B) 800 co-aggregates that contained 1.2×10^6 Sertoli cells and islet cells from 400 islets (n=7 mice). A total of 400 unmodified islets and 800 co-aggregates were transplanted into each mouse to ensure that the number of islet cells was equal in the grafts.

size of the co-aggregate was reduced by half so that each co-aggregate contained 1,500 Sertoli cells and islet cells from one half of an islet. A total of 400 unmodified islets or 800 co-aggregates that contained 1.2×10^6 Sertoli cells and islet cells from 400 islets were transplanted into each mouse to ensure that the number of islet cells was equal in the grafts. The recipient mice were not treated with any immunosuppressive therapy. Figure 3 shows the non-fasting blood glucose levels of the recipient mice before and after transplantation. When 400 unmodified BALB/c islets were transplanted, the blood glucose levels of all recipients were transiently normalized, but returned to the preoperative high level around 10 days after transplantation (Figure 3A). In contrast, when 800 co-aggregates were transplanted into seven mice, six of the seven demonstrated normoglycemia just 1 day after transplantation, and their blood glucose levels were maintained at levels lower than the preoperative levels for over 100 days. Notably, the blood glucose levels gradually became unstable over time (Figure 3B). These data indicate that Sertoli cells effectively protect the islet cells from rejection even though they are localized to the core region of the aggregates.

Plasma Insulin Levels and Glucose Tolerance in Transplantation Recipients.

Blood was taken fromthe recipientmice at various time points after intraportal transplantation of unmodified islets or coaggregates, and plasma insulin levels were determined by ELISA. The plasma insulin levels of normal C57BL/6 mice and STZ-induced diabetic mice were also determined as references (Figure 4). The plasma insulin levels in the blood of recipients of unmodified islets were transiently normal but declined rapidly to the same low level as in STZ-induced diabetic mice at 14 days post-transplantation. This indicates that the graft was lost because of rejection. The

plasma insulin levels of the recipients of co-aggregates declined gradually but were still higher 120 days post-transplantation than those of STZ-induced diabetic mice. These results indicate that islet cell damage was suppressed by the Sertoli cells in the co-aggregates.

To assess the blood glucose tolerance of recipients of co-aggregates, IPGTTs were performed 30 days post-transplantation. As shown in Figure 4B, the blood glucose levels of normal C57BL/6 mice and of the recipients of co-aggregates were highest 15

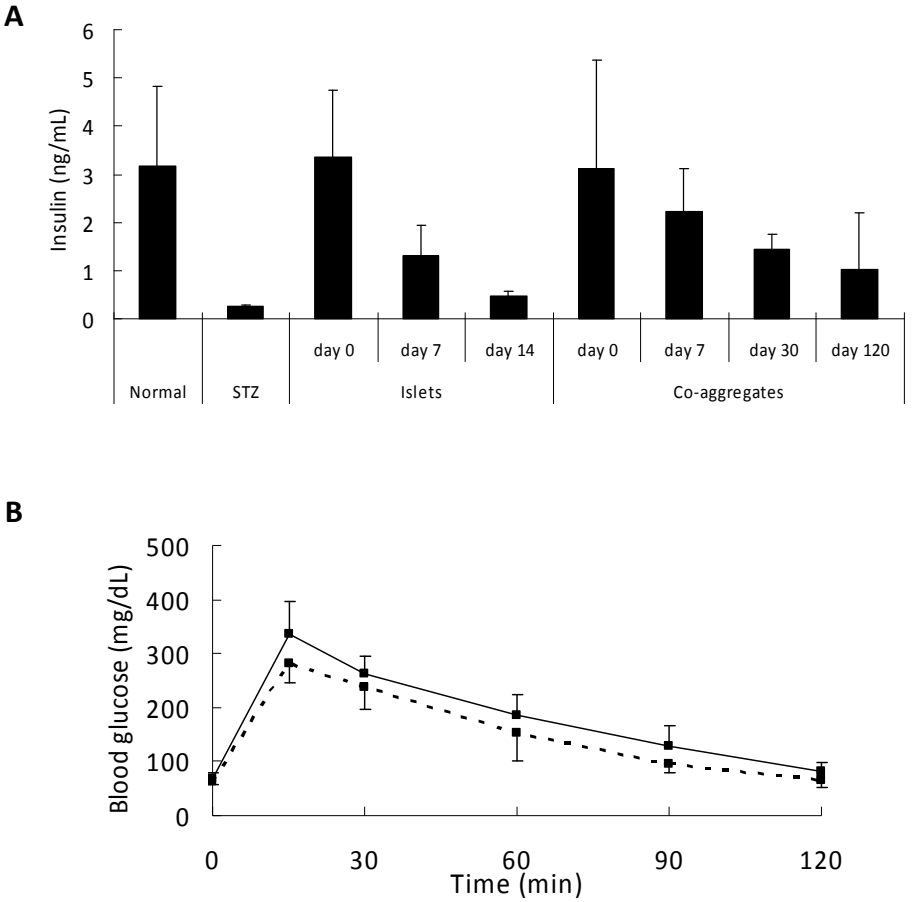
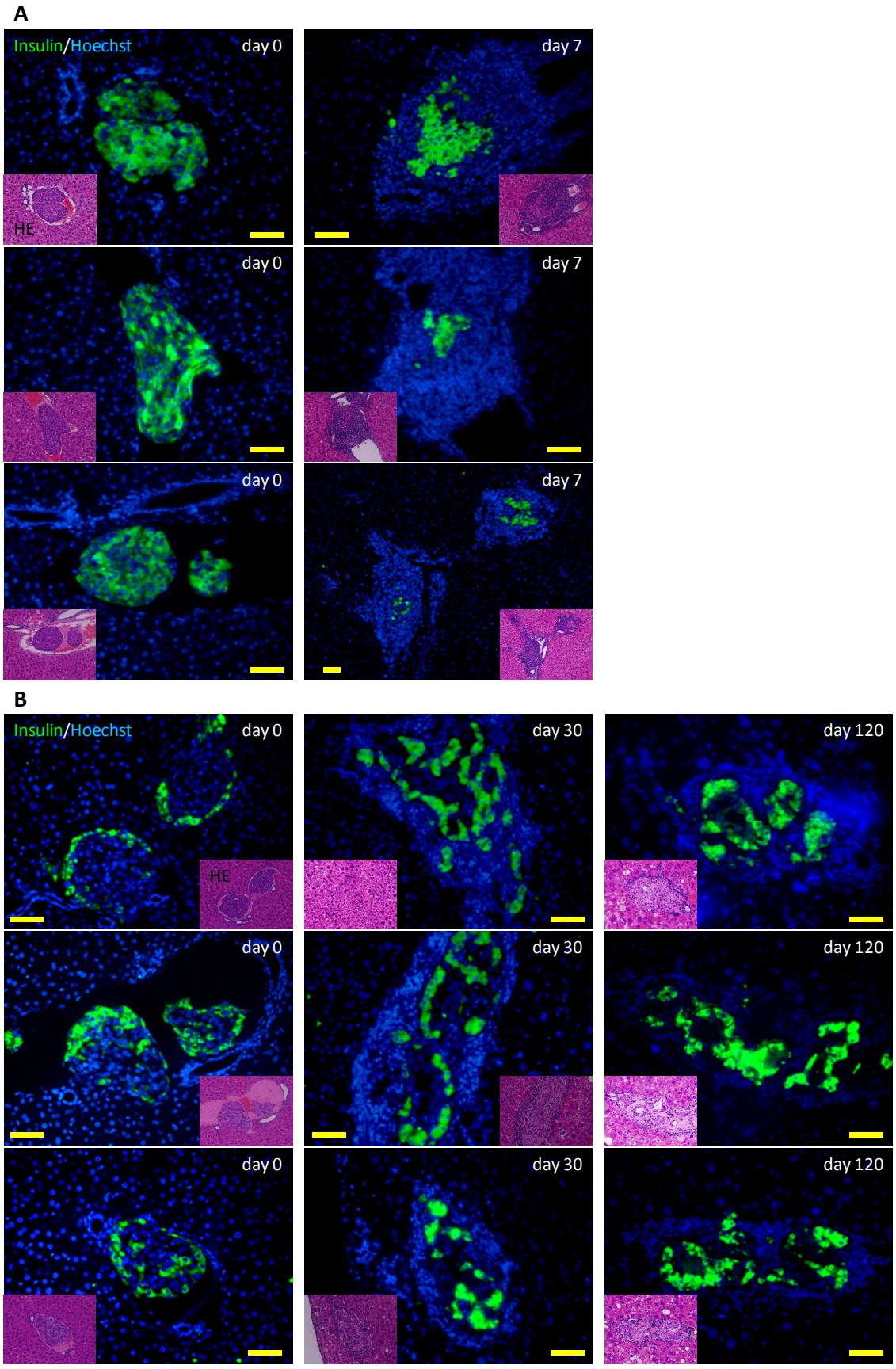


Figure 4. Graft function in the murine recipients of unmodified islets and co-aggregates that contained 1.2×10^6 Sertoli cells and islet cells from 400 islets. A, changes in blood insulin levels after intraportal transplantation of 400 unmodified islets and 800 co-aggregates. Normal C57BL/6 mice and STZ-induced diabetic mice were used as references. The blood insulin levels were determined by ELISA. B, intraperitoneal glucose tolerance tests performed 30 days after intraportal transplantation (n=6). Solid line: recipient mice of co-aggregates; dashed line, normal mice.

minutes after glucose injection. Afterward, blood glucose levels declined gradually over the following 120 minutes. No significant differences were observed in the changes in blood glucose levels between normal C57BL/6 mice and the recipients of co-aggregates. These data indicate that the islet cells of the co-aggregates allowed normal regulation of blood glucose levels even 30 days post-transplantation.

Histological Analyses of Liver with Grafts.

Livers were retrieved from recipient mice at various time points after transplantation of naive islets and coaggregates and subjected to histological analysis. Figure 5 shows images of thin sections of liver along with the naïve islets or co-aggregates. These tissue sections were stained with Alexa488-labeled anti-insulin antibodies (green fluorescence) and HE. When naive islets were transplanted, the islets were clearly seen in slices stained with HE and immunofluorescent staining just after transplantation, and insulin-positive cells were observed. However, 7 days post-transplantation, the insulin-positive cells were surrounded by lymphocytes (Figure 5A), and most of the insulin-positive cells had disappeared at 14 days post-transplantation. This indicates that the graft was recognized and attacked by the host immune system. In contrast, when co-aggregates were transplanted, there were insulin-positive cells at the periphery of the aggregates at day 0, and plenty of insulin-positive cells could be seen in the livers even 120 days post-transplantation (Fig. 5B) despite the infiltration of many lymphocytes into the grafts. These results indicate that allogeneic Sertoli cells in the aggregates exerted some protective effects on the graft and allowed the graft to survive for a long time in the liver despite the lack of systemic immunosuppression.


A
Insulin/Hoechst
day 0
day 7
HE
day 0
day 7
day 0
day 7
B
Insulin/Hoechst
day 0
day 30
day 120
HE
day 0
day 30
day 120
day 0
day 30
day 120

DISCUSSION

Some groups reported previously that the graft survival of islets could be improved by co-transplantation of the islets with Sertoli cells in allotransplantation models[8,9] and even in xenotransplantation models.[21] Korbutt *et al.* reported that islet graft survival could be improved without the use of immunosuppressive drugs by co-transplantation of the islets with Sertoli cells under the kidney capsule in a rat allotransplantation model.[8] Ramji *et al.* reported that xenogeneic islet graft survival could be prolonged by co-transplantation with porcine Sertoli cells in mice.[21] In all of these studies, islets and Sertoli cells were co-transplanted into the renal subcapsular site to co-localize these two kinds of cells. Although these methods gave promising results, the methods are not suitable for clinical islet transplantation. Specifically, islets and Sertoli cells are transfused into the liver via the portal vein. While Sertoli cells can be transported by blood flow into smaller veins, these same veins cannot accommodate the passage of the much larger islets. Thus, Sertoli cells could not be co-localized with the islets using this method. To overcome this problem, methods were examined for the co-localization of Sertoli cells and islets in the liver. In chapter 3, the use of single-stranded oligonucleotides, polyethylene glycol, and phospholipids allowed to localize Sertoli cells onto islet surfaces in the liver even after intraportal transplantation. The number of Sertoli cells is limited, however, because Sertoli cells cover the islets as a monolayer. In this chapter, another novel approach in which sufficient numbers of

Figure 5. Immunohistochemical analyses of islet grafts in mouse livers. Insulin staining (green) and hematoxylineosin staining (inset) of liver grafts. The nuclei were stained with Hoechst 33258 (blue). A, 400 unmodified islets shown 0 and 7 days after intraportal transplantation. B, 800 co-aggregates that contained 1.2×10^6 Sertoli cells and islet cells from 400 islets in a mouse liver shown 0, 30, and 120 days after intraportal transplantation. Scale bar: 100 μm.

Sertoli cells were incorporated into a co-aggregate with islet cells *in vitro* before transplantation, were tried.

Sertoli cells are connected to each other via tight junctions[22] that make up the blood-testis barrier[22] which inhibits the passage of large molecules, such as antibodies and white blood cells, into the seminiferous tubules. It was expected that this effect would protect islet cells from the host immune system. Sertoli cells, however, occupied the core part of co-aggregates, while islet cells engulfed the core aggregate of Sertoli cells by the simple hanging drop method as shown in Figure 1C. Although several attempts, such as sequential aggregate formation in which islet cells formed an aggregate first and then Sertoli cells were added to the aggregate, were examined, coverage of an islet cell aggregate with Sertoli cell layers could not be realized. It reported that different kinds of cells spontaneously sorted into separate areas in co-aggregates.[23,24] These observations were explained by the cell-type-dependent interfacial energy[25] and numerically reproduced by the cellular Potts model.[26] In case of mixing the higher-interfacial energy cells and the lower-interfacial energy cells, a core part was occupied by a cluster of cells with the higher-interfacial energy and is covered by a layer of the lower-interfacial energy cells.[25,26] It is hardly expected that the Sertoli cell barrier protect islet cells from the attack by the host immune system. As shown in Figures 3 and 5, however, existence of Sertoli cells in the core part of co-aggregates allowed long-term islet graft survival in mouse diabetes liver. Other protection effects by Sertoli cells should play a role.

The cellular immune response by T lymphocytes is impeded by the Fas-Fas ligand system.[27,28] Fas (CD95) is a membrane receptor that induces cellular after binding of the Fas ligand.[29,30] Activated T lymphocytes express Fas, and Sertoli cells

express the Fas ligand; further, Sertoli cells can eliminate Fas-positive activated T lymphocytes.[11] Sertoli cells produce other immune-modulating factors, such as transforming growth factor (TGF)-β[12] and clusterin.[13] These factors might play a role in the long-term survival of allogeneic grafts and co-aggregates of islet cells and Sertoli cells in the livers of diabetic mice.

Although the mechanism by which Sertoli cells protect islet cells from rejection remains unclear for now, the approach used in this chapter results in successful co-localization of Sertoli cells and islet cells in the liver and is quite practical for clinical islet transplantation.

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Chapter 5

Co-aggregates of Regulatory T Cells and Islet Cells Allow Long-term Graft Survival in Liver Without Immunosuppression

INTRODUCTION

Transplantation of islets of Langerhans (islets) is a promising to treat patients with insulin-dependent diabetes mellitus (type I diabetes). More than 300 type I diabetes patients are treated with allogeneic islet transplantation each year. Immunosuppressive drugs are administered to the patients to inhibit rejection of the islet graft. Although the introduction of a steroid-free immunosuppressive protocol has greatly improved the outcomes of islet transplantation,[1] lifelong administration of immunosuppressive drugs is expected to cause various side effects, such as infection, malignant tumor formation, and impairment of islet functions.[2] Various studies have sought alternatives to immunosuppressive drugs, including the bioartificial pancreas[3] in which islets are enclosed within a semi-permeable membrane,[4,5] radiation of islets with ultraviolet light,[6] islet culture under low-temperature conditions prior to transplantation,[7] and systemic infusion of regulatory T (Treg) cells.[8]

Treg cells are indispensable for the maintenance of dominant self-tolerance and immune homeostasis.[9] Their dysfunction causes various autoimmune diseases, immunopathology, and allergy.[10] Treg cells (most of which are $CD4^+$ T cells that

express CD25) can suppress the activation, proliferation, and effector functions of a wide range of immune cells *in vitro* and *in vivo*, including $CD4^+$ and $CD8^+$ T cells, natural killer (NK) and NKT cells, B cells, and antigen-presenting cells (APCs).[10] Some groups have reported that Treg cells can control allo- and autoimmunity and can also be used successfully for adoptive transfer in many animal models.[11–14] The ability of systemic administration of Treg cells to control graft rejection in islet transplantation has been investigated, and results have demonstrated that Treg cells might be able to prevent allograft rejection.[8] However, venous infusion of Treg cells increases systemic Treg revels, and might be associated with risks of systemic immunologic unresponsiveness, opportunistic infections, and neoplasm formation.

In this chapter, co-aggregates of $CD4^+CD25^+$ Treg cells and islet cells were prepared, and the co-aggregates were infused into liver through the portal vein. Treg cells in co-aggregates were expected to coexist with islet cells and become actively involved in the creation of an immune-privileged site. The protective effects of Treg cells in co-aggregates of BALB/c islet cells and C57BL/6 Treg cells were examined using streptozotocin (STZ)-induced diabetic C57BL/6 mice as recipients.

EXPERIMENTAL PROCEDURES

Materials.

The following reagents were purchased from the indicated suppliers: Blocking One, trypsin, penicillin-streptomycin mixed solution, 2-mercaptethanol (2-ME), and STZ from Nacalai Tesuque (Kyoto, Japan); Hoechst 33258 nuclear stain from Dojindo

Laboratories (Kumamoto, Japan); Alexa 488-labeled goat anti-guinea pig IgG, Hanks' balanced salt solution (HBSS), and RPMI 1640 from Invitrogen (Carlsbad, CA, USA); fetal bovine serum (FBS) from Equitech-Bio, Inc. (Kerrville, TX, USA); phosphate-buffered saline (PBS) from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan); enzyme-linked immunosorbent assay (ELISA) kits for the mouse insulin assay from Shibayagi Co., Ltd. (Gunma, Japan); polyclonal guinea pig anti-swine insulin from Dako (Glostrup, Denmark); formalin solution, Tween 20, and Triton X-100 from Wako Pure Chemical (Osaka, Japan); red blood cell lysing buffer from Sigma-Aldrich (St. Louis, MO, USA); goat affinity-purified antibody to rat IgG (Whole Molecule) from MP Biomedicals (Solon, OH, USA); biotin rat anti-mouse CD25, PE streptavidin, and FITC rat anti-mouse CD4 from BD Biosciences (San Jose, CA, USA); anti-PE MicroBeads from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany); and collagenase from Nitta Gelatin (Osaka, Japan).

Rat anti-CD8 (3.155) and rat anti-CD24 (J11d) were kindly provided by Professor Shimon Sakaguchi.

Preparation of $CD4^+CD25^+$ Treg Cells and Islet Cells.

The Kyoto University Animal Care Committee approved all animal experiments. $CD4^+CD25^+$ Treg cells were isolated from C57BL/6 or enhanced green fluorescent protein (EGFP)-transgenic C57BL/6 mice (8-week-old male mice, Japan SLC, Inc., Shizuoka, Japan) using magnetic-activated cell sorting (MACS; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Briefly, spleen and lymph node cell suspensions prepared from C57BL/6 or EGFP-transgenic C57BL/6 mice and enriched for $CD4^+$ cells by panning; anti-rat IgG-coated dishes were used to remove $CD8^+$ or $CD24^+$ cells

treated with rat anti-CD8 and rat anti-CD24 from the cell suspensions. These cells were treated with biotin rat anti-mouse CD25, PE streptavidin, and anti-PE MicroBeads, and sorted by MACS. The sorted cells were used immediately in the following experiments.

To examine the purity of MACS-sorted $CD4^+CD25^+$ Treg cells, the cells were treated with FITC rat anti-mouse CD4 and analyzed using a fluorescence-activated cell analyzer (Guava EasyCyte Mini; Millipore, Billerica, MA, USA).

Islets were isolated from BALB/c mice (7-week-old male mice, Japan SLC, Inc., Shizuoka, Japan) using the collagenase digestion method.[15] Briefly, a collagenase solution (0.5 mg/mL in HBSS) was injected into each mouse pancreas through the bile duct. The pancreas was then collected from the mouse and incubated for 18 minutes at 37°C. After the digested tissue had dissociated into pieces, the tissue was washed with HBSS, and islets were isolated from the digestion reaction using Ficoll density gradient purification. Isolated islets were cultured for 2 days in RPMI 1640 medium with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin to remove cells damaged by the isolation procedure.

Islets were disassociated into single cells by incubation in 1 mL trypsin solution (500 μg/mL in HBSS) at 37°C for 2 minutes, followed by the addition of 1 mL of medium and pipetting with a Pasteur pipette to apply mechanical force. Resultant single cells were resuspended in RPMI 1640 medium with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 50 μM 2-ME.

Co-aggregation of Treg Cells and Islet Cells.

Co-aggregates of Treg cells and islet cells were prepared on agarose hydrogel with small round-bottomed wells. A mold (Microtissues Inc., Providence, RI, USA) was

used to create the hydrogel with 256 (16 × 16) wells, each 250 μm in diameter. Hot 2.5% agarose solution in PBS (50-90°C) was injected into the mold and chilled to form a gel on ice. The hydrogel was equilibrated in RPMI 1640 medium supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 50 μM 2-ME. Immediately after isolation, Treg cells were suspended in 100 μL RPMI 1640 medium at a density of 3000 cells per well. Cells that were obtained by the dissociation of 256 islets were suspended in 100 μL of RPMI 1640 medium with 10% FBS. These suspensions of Treg cells and islet cells were mixed together. The cell suspension (200 μL) was applied to the hydrogel to achieve a final concentration of approximately 3000 Treg cells and islet cells equivalent to one islet per well. Cells were cultured on the hydrogel for 4 days at 37°C in a humidified incubator in an atmosphere of 5% CO_2/95% air. Treg cells isolated from EGFP-transgenic mice were used to examine the distribution of Treg cells in co-aggregates during co-aggregate formation and for long-term *in vitro* culture. The co-aggregates were observed under a fluorescence microscope (IX71, Olympus Optical, Co., Ltd., Tokyo, Japan) and a confocal laser scanning microscope (FluoView, FV10i, Olympus Optical Co.).

***In Vitro* Glucose Stimulation Test for Co-aggregates.**

Fifty co-aggregates (each composed of 3000 Treg cells and islet cells equivalent to one islet) were exposed sequentially to solutions of 0.1 g/dL, 0.3 g/dL, and 0.1 g/dL glucose in Krebs-Ringer's buffer (KRB); they were incubated for 1 hour at 37°C in each solution. The supernatants were collected, and the insulin concentrations of each solution were determined using ELISA according to the manufacturer's instructions. The glucose stimulation test was also performed using 50 unmodified islets as a

reference.

Intraportal Transplantation of Co-aggregates.

Eight-week-old male C57BL/6 mice were used as the co-aggregate transplant recipients. Diabetes was induced in C57BL/6 mice with a single intraperitoneal injection of STZ (120 mg/kg body weight in citrate buffer, pH 4.2) 1 week before transplantation. Mice were used as diabetic recipients when their plasma glucose levels exceeded 450 mg/dL in two consecutive measurements. The diabetic mice were anesthetized during surgery by mask inhalation of isoflurane using a specialized instrument (400 Anesthesia Unit, Univentor, Malta); the isoflurane concentration was 4.5% to 5.0% for induction and 2.0% for maintenance (airflow rate, 200 mL/min).

A suspension of 400 co-aggregates containing 1.2×10^6 Treg cells isolated from C57BL/6 mice and islet cells equivalent to 400 BALB/c mouse islets was infused into the portal vein and the co-aggregates plugged small vessels in the liver. As a control experiment, 400 unmodified BALB/c mouse islets were also infused through the portal vein. A total of 400 unmodified BALB/c mouse islets or 400 co-aggregates that contained 1.2×10^6 Treg cells isolated from C57BL/6 mice and islet cells equivalent to 400 BALB/c mouse islets were transplanted into each C57BL/6 mouse to ensure that the number of islet cells was equal in the grafts. Plasma glucose levels were monitored daily during the initial 30 post-transplantation days and subsequently monitored every 2 days. The graft survival periods were defined from the day of transplantation to the day that the first of two consecutive plasma glucose levels >250 mg/dL was recorded. Blood was collected from recipients and centrifuged to obtain plasma. Plasma insulin levels were determined using ELISA according to the manufacturer's instructions. Recipients

were subjected to intraperitoneal glucose tolerance tests (IPGTTs) 90 days post-transplantation to evaluate glucose tolerance.

Histochemical Analysis.

Some recipient mice were sacrificed at predetermined times after transplantation of the co-aggregates or unmodified islets. The livers were retrieved and immersed in 4% formalin solution for 2 days at room temperature. After the formalin solution was removed, the livers were dehydrated in alcohol solutions and embedded in paraffin. The livers were sliced into sections 4 μm in thickness. After deparaffinization, the sections were permeabilized with 0.2% Triton X-100 in PBS at room temperature for 15 minutes. The specimens were then treated with Blocking One for 1 hour followed by washing with PBS. Sections were treated with 1% polyclonal guinea pig anti-swine insulin in Blocking One for 1 hour at room temperature. Sections were then incubated with fluorescently labeled secondary antibody (0.2% Alexa 488-labeled goat anti-guinea pig IgG in Blocking One) at room temperature for 1 hour followed by washing with PBS containing 0.05% Tween-20. The stained specimens were observed under a fluorescence microscope (BX51, Olympus Optical Co., Ltd., Tokyo, Japan). The specimens were also stained with hematoxylin-eosin (HE) using a conventional staining protocol.

RESULTS

Co-aggregate Formation and Morphology.

Treg cells were isolated from the spleens and lymph nodes of C57BL/6 mice using MACS. A fluorescence-activated cell analyzer was used to evaluate cell purity. Approximately 95% of the MACS-sorted cells were CD4-positive and CD25-positive (Figure 1A). Islets were isolated from BALB/c mice (Figure 1B). The islets were separated into single cells by treatment with trypsin. Their viability after trypsin treatment was >93% as estimated by the trypan blue exclusion method.

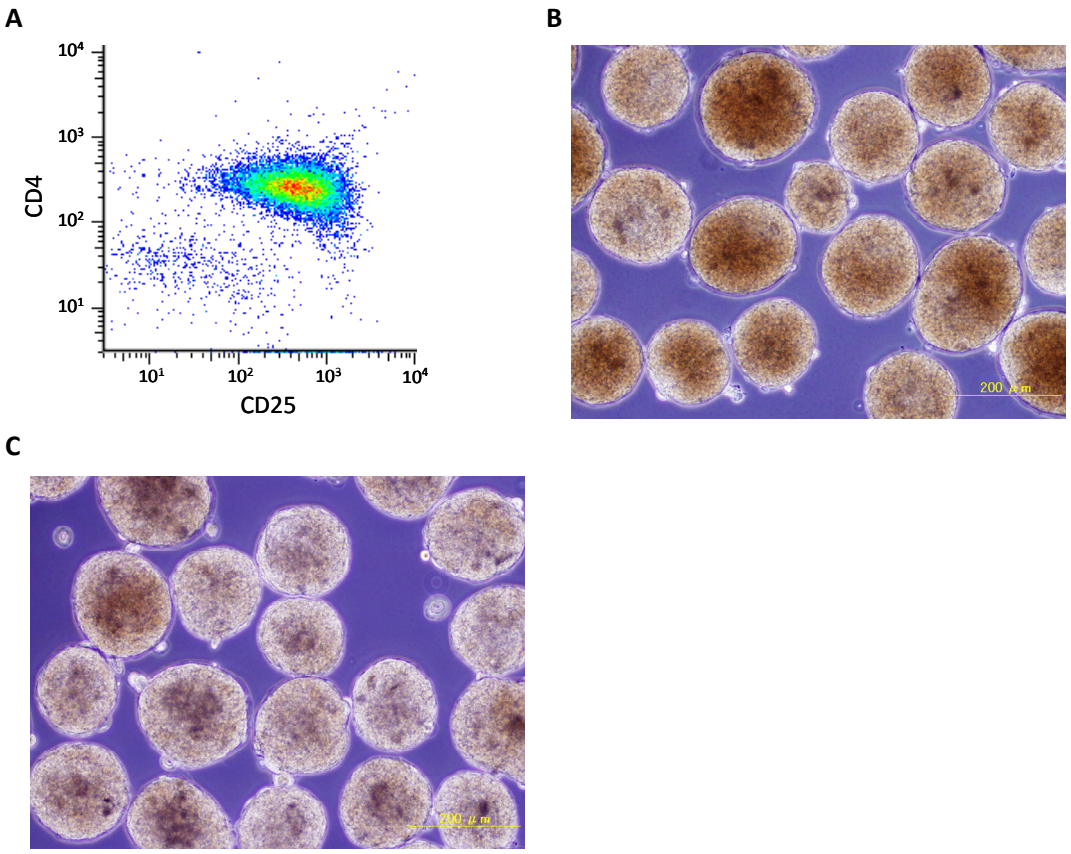

Figure 1. Co-aggregates of Treg cells and islet cells. (A) Magnetic-activated cell-sorted Treg cells analyzed using a fluorescence-activated cell analyzer immediately after isolation from C57BL/6 mice. (B) A phase contrast microscopic image of islets that were cultured for 2 days after isolation from BALB/c mice. (C) A phase contrast microscopic image of co-aggregates of Treg cells and islet cells after 4 days in culture.

A mixed suspension of Treg cells and islet cells were applied to a hydrogel with small wells and cultured. Each well contained 3000 Treg cells and islet cells equivalent to one islet. Co-aggregates were formed after 4 days in culture (Figure 1C). The time course of co-aggregate formation from islet cells and EGFP-Treg cells is depicted in Figure 2. Treg cells and islet cells were randomly mixed on the first day. They gathered into round aggregations during the initial 2 days of culture. These two cell types formed spherical aggregates and the co-aggregates steadily became more compact during the following 4 days. The two different kinds of cells were located randomly in the aggregates (Figure 2), and still coexisted in the aggregates after 4 weeks in culture.

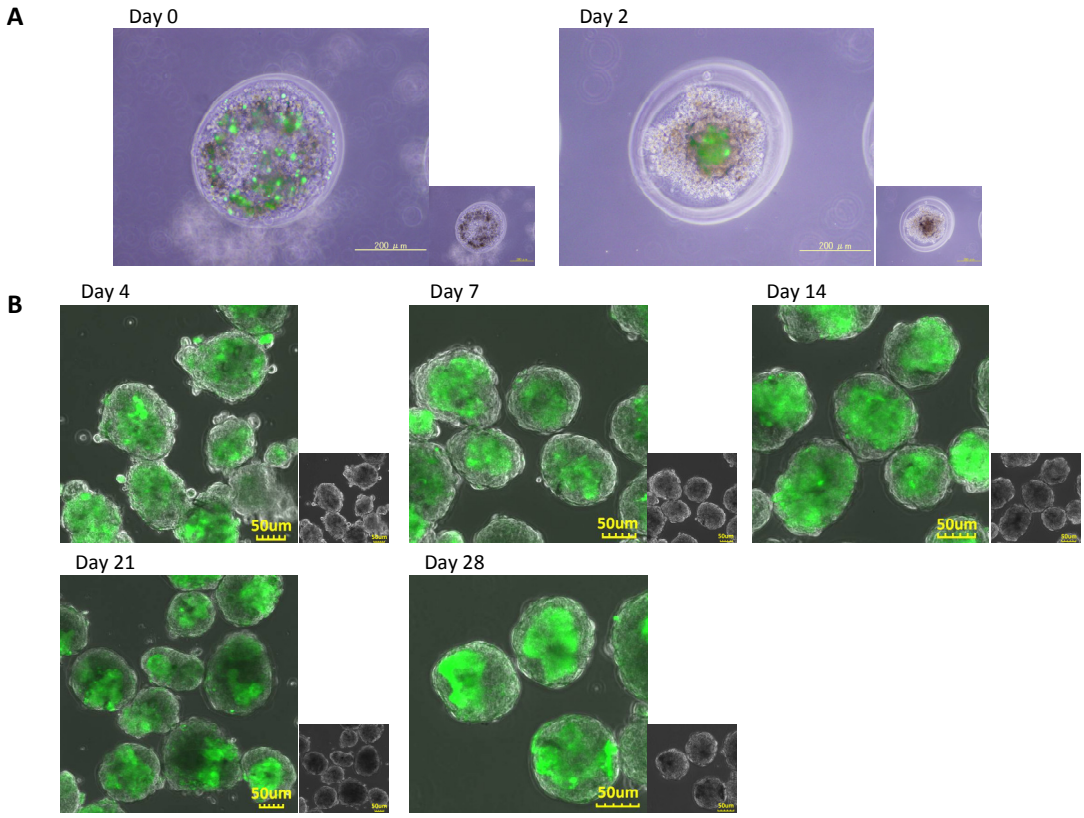

Figure 2. Time course of co-aggregate formation from populations of Treg cells and islet cells. (A) A mixed suspension of EGFP-Treg cells and islet cells was applied to small wells made of agarose hydrogel. The small wells were observed at day 0 and day 2 under a fluorescence microscope. (B) Co-aggregates of EGFP-Treg cells and islet cell were observed at the indicated days under a confocal laser scanning fluorescent microscope.

Co-aggregates of Sertoli cells and islet cells were prepared in chapter 4; Sertoli cells occupied the core of co-aggregates, while islet cells occupied the periphery. In contrast to that result, Treg cells and islet cells were distributed randomly throughout co-aggregates (Figure 2). Sertoli cells are adherent cells and are intimately connected to each other through tight junctions,[16] while Treg cells are floating cells and do not spontaneously agglutinate with each other. Cell-cell interaction determines the distribution of each cell type in the aggregates.

In Vitro Insulin Secretion from Co-aggregates.

Co-aggregates were subjected to a glucose stimulation test to determine whether they are able to control insulin secretion in response to changes in glucose levels. When the glucose concentration was increased from 0.1 g/dL to 0.3 g/dL, both co-aggregates and unmodified islets increased levels of insulin release compared with basal levels

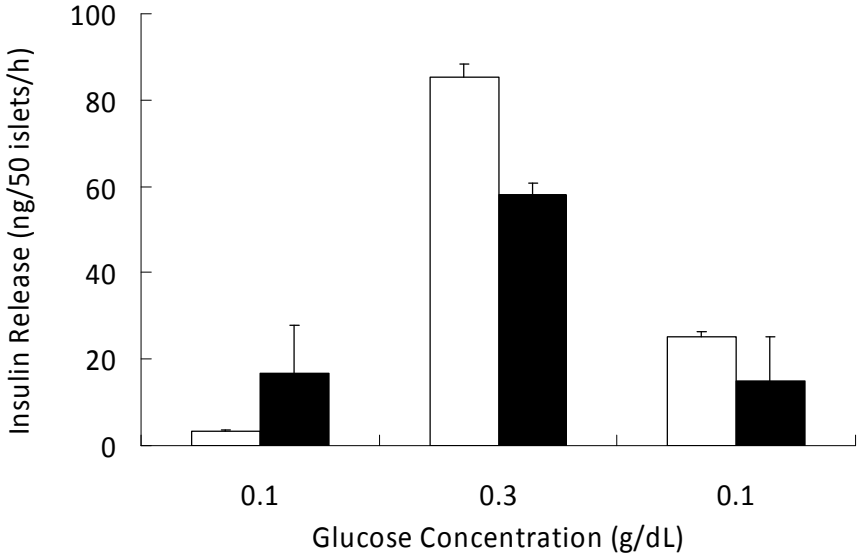

Figure 3. *In vitro* glucose stimulation test. Unmodified islets and co-aggregates were exposed sequentially for 1 hour each to 0.1, 0.3, and 0.1 g/dL glucose in Krebs-Ringer buffer (KRB) at 37°C. The values for the released insulin are expressed as mean ± SD for n = 4 cultures. White bars, islets; Black bars, co-aggregates.

(Figure 3). When the glucose concentration was returned to 0.1 g/dL after exposure to 0.3 g/dL glucose, insulin release returned to basal levels. The levels of insulin released in response to glucose did not differ significantly between co-aggregates and unmodified islets. These results indicate that islet cells in the co-aggregates maintained their ability to regulate insulin release in response to changes in glucose levels.

Transplantation of Co-aggregates into the Livers of STZ-Induced Diabetic Mice.

Either 400 unmodified islets or 400 co-aggregates that contained 1.2×10^6 Treg cells and islet cells from 400 islets were transplanted into each STZ-induced diabetic C57BL/6 mouse (n = 9 per group) to ensure that the grafts contained equal numbers of islet cells. The recipient mice were not treated with any immunosuppressive therapy. Non-fasting blood glucose levels of the recipient mice before and after transplantation are summarized in Figure 4. When 400 unmodified BALB/c islets were transplanted, the blood glucose levels of 8 of the 9 control recipients were transiently normalized, but returned to the preoperative high level approximately 10 days after transplantation (Figure 4A). In contrast, when 400 co-aggregates were transplanted, six of the nine experimental recipients demonstrated normoglycemia just 1 day after transplantation, and their blood glucose levels were maintained at levels below the preoperative levels for >100 days (Figure 4B). The graft survival periods were defined from the day of transplantation to the day on which the first of two consecutive plasma glucose levels >250 mg/dL was recorded. Graft survival was plotted versus the number of post-transplantation days (Figure 4C). These data suggest that Treg cells in the aggregates are able to effectively protect the islet cells from rejection.

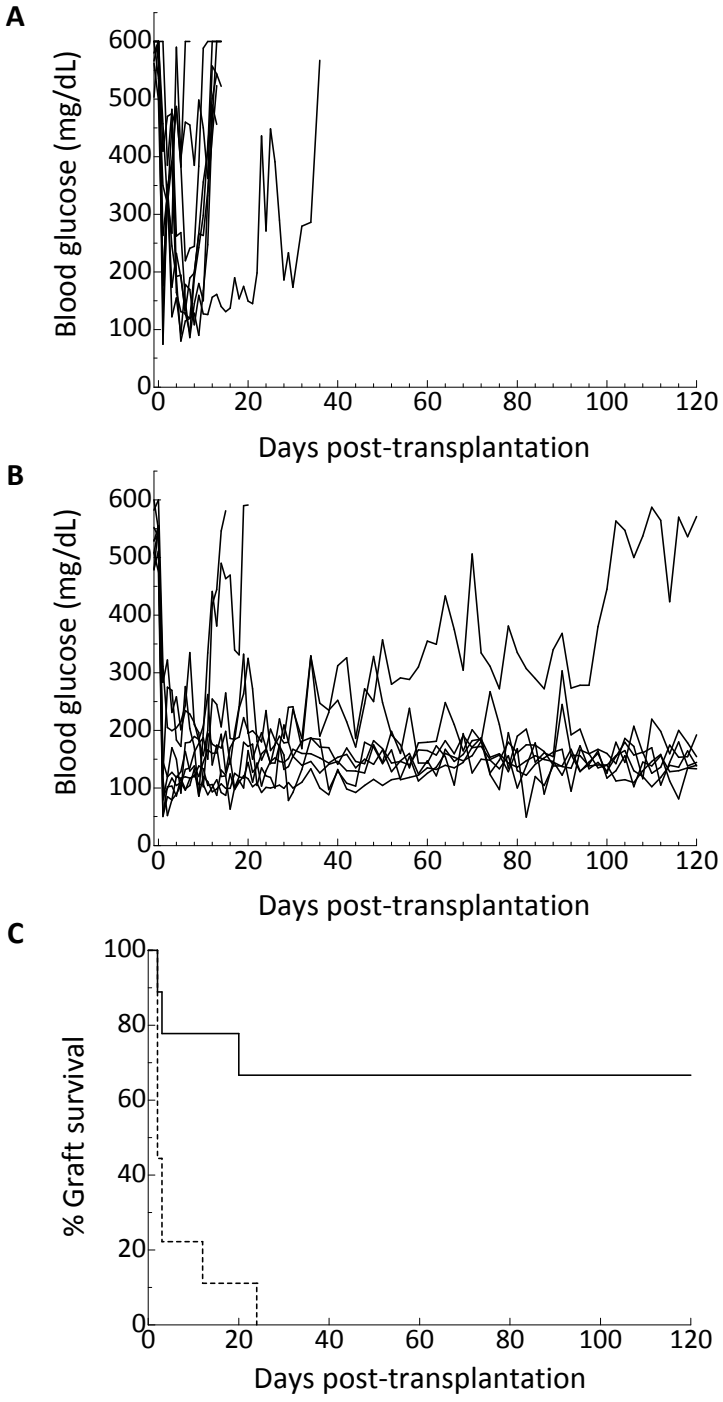

Figure 4. Non-fasting blood glucose levels of STZ-induced diabetic C57BL/6 mice after intraportal transplantation. (A) After transplantation of 400 unmodified BALB/c mouse islets (n = 9 mice). (B) After transplantation of 400 co-aggregates, each of which contained 1.2×10^6 Treg cells isolated from C57BL/6 mice and islet cells from 400 BALB/c mouse islets (n = 9 mice). (C) Kaplan-Meier survival curves for unmodified islets (dashed line) and co-aggregates (solid line). They were compared using the log-rank test ($p < 0.0015$). The day of graft rejection was defined as the day on which the first of two consecutive plasma glucose measurements >250 mg/dL was recorded.

Plasma Insulin Levels and Glucose Tolerance in Recipients.

Blood samples were obtained from recipient mice at predetermined days after the intraportal transplantation of unmodified islets or co-aggregates, and their plasma insulin levels were determined using ELISA. The plasma insulin levels of wild-type C57BL/6 mice and STZ-induced diabetic mice were also determined as references (Figure 5A). The plasma insulin levels of the recipients of unmodified islets at 14 days post-transplantation (the population in which blood glucose levels had returned to the

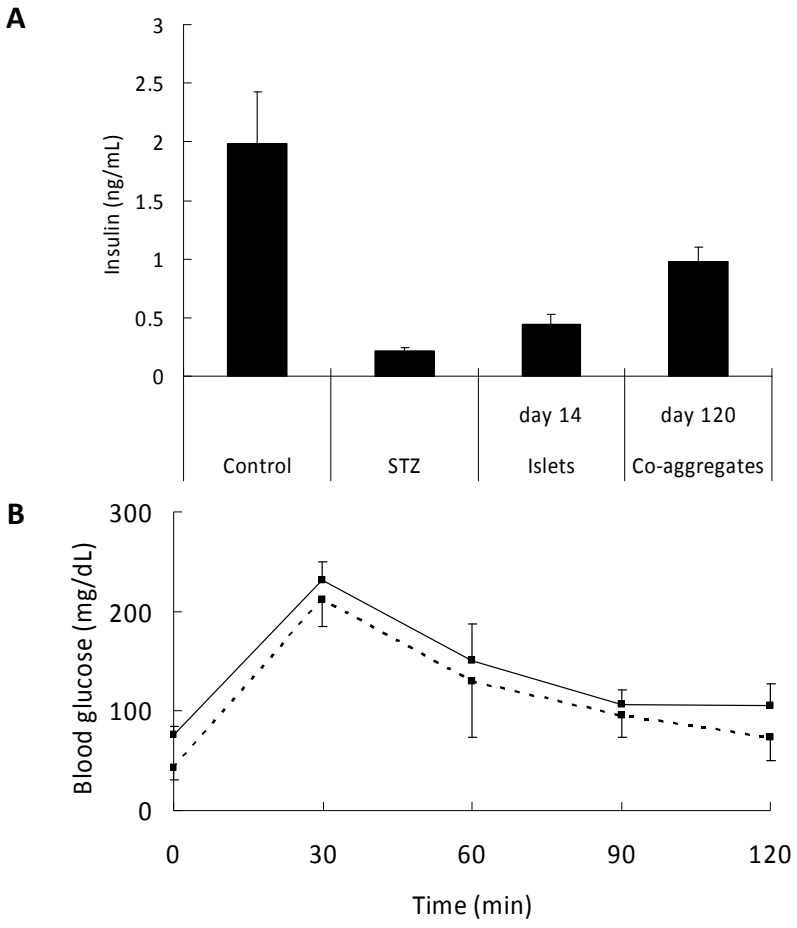

Figure 5. Plasma insulin levels and intraperitoneal glucose tolerance tests of transplant recipients. (A) Plasma insulin levels of wild-type C57BL/6 mice (Control), STZ-diabetic mice (STZ), and recipients of 400 unmodified islets (Islets) or 400 co-aggregates (Co-aggregates). (B) Intraperitoneal glucose tolerance tests performed 90 days after intraportal transplantation (n = 4 mice). Solid line, recipients of 400 co-aggregates; dashed line, wild-type C57BL/6 mice.

preoperative high level) were comparable to the low plasma insulin levels observed in STZ-induced diabetic mice, indicating loss of the graft. At 120 days post-transplantation, plasma insulin levels of the co-aggregate recipients were approximately one-half of the levels observed in wild-type mice, but were higher than the levels observed in STZ-induced diabetic mice. These results indicate that islet cells in the co-aggregates were still functioning and secreting insulin.

IPGTTs were performed 90 days post-transplantation to assess the blood glucose tolerance of co-aggregate recipients. The blood glucose levels of wild-type C57BL/6 mice and co-aggregate recipients were highest 30 minutes after glucose injection (Figure 5B). Afterward, blood glucose levels declined gradually over the following 120 minutes. No significant differences were observed in the changes in blood glucose levels between wild-type C57BL/6 mice and co-aggregate recipients. These data indicate that the islet cells of the co-aggregates allowed normal regulation of blood glucose levels even 90 days post-transplantation.

Histological Analysis of Islet Grafts in Liver.

Livers were retrieved from recipient mice at predetermined time points after transplantation and subjected to histological examination. Figure 6 depicts microscopic images of thin sections of the liver with unmodified islets or co-aggregates. These tissue sections were stained with Alexa488-labeled anti-insulin antibodies (green fluorescence) and Hoechst 33258 (blue). The inserts show these tissue sections stained with HE. When unmodified islets were transplanted, insulin-positive cells could be seen; however, their numbers decreased and they were surrounded by many lymphocytes at 7 days post-transplantation (Figure 6A). Insulin-positive cells were rarely detected at 14 days

post-transplantation. These findings indicate that the allogeneic unmodified islets were rejected by the host immune system. In contrast, when co-aggregates were transplanted, a number of insulin-positive cells were observed in the livers as late as 120 days post-transplantation (Figure 6B), and no lymphocytic infiltration was observed surrounding the co-aggregates. These results indicate that Treg cells in the aggregates exerted to some extent protected the graft from attack by the host immune system, and thus allowed the graft to survive for a long time in the liver even without administration of immunosuppressive drugs.

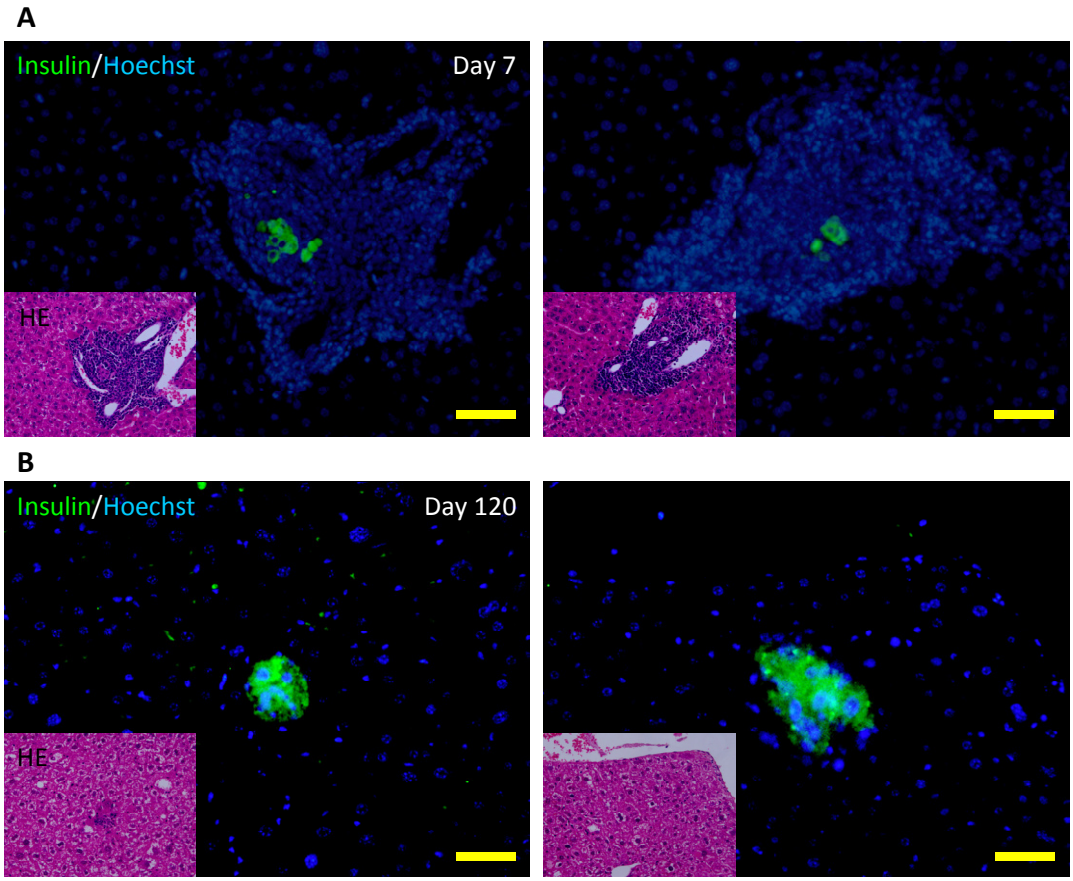

Figure 6. Immunohistochemical examination of islet grafts in C57BL/6 mouse livers. Thin tissue sections were stained with Alexa488-labeled anti-insulin antibodies (*green*) and Hoechst 33258 (*blue*). The inserts show these tissue sections stained with hematoxylin-eosin (HE). (A) 7 days after intraportal transplantation of unmodified BALB/c mouse islets. (B) 120 days after intraportal transplantation of co-aggregates of Treg cells isolated from C57BL/6 mice and islet cells from BALB/c mice. Scale bar: 100 μm.

DISCUSSION

Chapter 4 examined the intrahepatic transplantation of co-aggregates of islet cells and Sertoli cells, which play a crucial role in creating the immunoprivileged environment in the testis.[17] The co-aggregation of these two cell types allowed long-term allogeneic islet graft survival in the liver without immunosuppression. However, the source of Sertoli cells (testis) is limited, and thus they are rarely obtained in the clinical setting. The strong immune-suppressive functions of Treg cells are known, and they can be isolated from peripheral blood in sufficient numbers.[9,10] Some groups have reported the use of Treg cell transfusion to successfully effect adoptive transfer in many animal models.[8,11–14] In islet transplantation, the transfusion of Treg cells promotes tolerance of allogeneic islet grafts in animal models.[8] Battaglia *et al.* reported[8] long-term islet graft survival in STZ-induced diabetic BALB/c mice that received islets from C57BL/6 mice under the kidney capsule; a Treg cell-enriched T-cell population (5×10^6 Treg cells per graft) isolated from BALB/c mice had been injected the day before islet transplantation. However, the systemic infusion of a large number of Treg cells might cause a variety of side effects, including opportunistic infections and regeneration of neoplasm. To overcome these problems, co-aggregates of Treg cells and islet cells were employed to decrease the number of Treg cells to 1.2×10^6 cells per graft and to localize Treg cells near the islet cells even when the islet cells were transfused through the portal vein into the liver as in clinical islet transplantation. Although only one-fourth as many Treg cells per graft as Battaglia *et al.* were employed, in this experience they effectively protected allogeneic islets from graft rejection.

There have been several attempts to localize Treg cells near islets in the context of

intrahepatic islet transplantation.[18] Marek *et al.* previously demonstrated[18] the ability to bind Treg cells to islet surfaces using biotin-poly(ethylene glycol)-N-hydroxysuccinimide ester (biotin-PEG-NHS) and streptavidin as binding molecules. They reported that islets maintain their viability and insulin secretion function after Treg immobilization, and that Treg cells exert some protective activity on the islets against immune attack *in vitro*. However, their *in vivo* efficacy has not yet been reported. Equal numbers of Treg cells and islet cells were used to prepare co-aggregates; therefore, the number of Treg cells per islet in this chapter is much larger than in Marek's study. This difference in the number of Treg cells may be the reason why we observed a clear immune-protective effect of Treg cells on allogeneic islet cell grafts (Figures 4-6).

The approach used in this chapter successfully co-localized Treg cells and islet cells in the liver and demonstrated long-term graft survival. It is believed that this method will be useful to realize immunosuppressive drug-free islet transplantation.

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SUMMARY

Chapter 1

Fibrinolytic urokinase (UK) can be conjugated on the surfaces of islets of Langerhans (islets) through DNA hybridization using single-stranded oligonucleotides (ssDNA) and poly(ethylene glycol)-conjugated phospholipid (ssDNA–PEG-lipid). The activity of UK was maintained on the islet surface, and the surface modifications did not influence islet morphology or islet ability to secrete insulin in response to changes in glucose concentration. It is believed that UK on islets can inhibit instant blood coagulation-mediated inflammatory reactions, thus suppressing islet graft loss at the early stage of islet transplantation.

Chapter 2

Liposomes loaded with vitamin E can be immobilized through DNA hybridization on human umbilical vein endothelial cells (HUVECs) using ssDNA–PEG-lipids, and liposomes are effectively internalized into cells. Vitamin E-loaded liposomes successfully protect cells from the oxidative stress induced by antimycin A. It is believed that vitamin E-liposomes effectively protect the endothelial cells of a donated organ from reperfusion damage.

Chapter 3

Sertoli cells, which are important for creating the immunoprivileged environment of the testis, can be immobilized on the surface of pancreatic islets through DNA hybridization using ssDNA–PEG-DPPE. Such immobilization did not influence islet

morphology or islet ability to secrete insulin. Importantly, the function of Sertoli cells was maintained on the islet surface. After intraportal transplantation of Sertoli cell-immobilized islets, the Sertoli cells were attached to the surface of islets in the liver. It is believed that this method will improve the success rate of islet transplantation.

Chapter 4

The hanging drop method is suitable for preparing aggregates of Sertoli and islet cells for transplantation. The core of the aggregates contained mainly Sertoli cells, while islet cells populated the periphery. Co-aggregates retained the functions of both Sertoli and islet cells. Notably, transplantation of these allogeneic co-aggregates into mice with chemically-induced diabetes via the portal vein resulted in long-term graft survival without systemic immunosuppression. This approach results in successful co-localization of Sertoli cells and islet cells in the liver and is quite practical for clinical islet transplantation.

Chapter 5

Regulatory T (Treg) cells, most of which are $CD4^+$ T cells that express CD25, can suppress the activation, proliferation, and effector functions of a wide range of immune cells. Co-aggregates of $CD4^+CD25^+$ Treg cells from C57BL/6 mice and islet cells from BALB/c mice were prepared on agarose hydrogel with small round-bottomed wells. Treg cells and islet cells were distributed randomly in the co-aggregates. After intraportal transplantation of the co-aggregates, Treg cells in the aggregates enabled the long-term survival of allogeneic islet cell grafts in the liver without the use of immunosuppressive drugs. It is believed that this method will be useful to realize

immunosuppressant-free islet transplantation.

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Kyoto

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