Title

PROTECTION OF ISLETS OF LANGERHANS FROM COMPLEMENT MEDIATED CYTOTOXICITY

Author(s)

NGUYEN MINH LUAN

Citation

Kyoto University (京都大学)

Issue Date

2011-09-26

URL

https://doi.org/10.14989/doctor.k16406

Type

Thesis or Dissertation

Textversion

author

Kyoto University
PROTECTION OF ISLETS OF LANGERHANS FROM COMPLEMENT MEDIATED CYTOTOXICITY

NGUYEN MINH LUAN

2011
Dedicated to

My parents and my wife
CONTENTS

GENERAL INTRODUCTION .................................................................................. 1
REFERENCES ........................................................................................................... 7

CHAPTER 1

Immobilization of the soluble domain of human complement receptor 1 (sCR1) on agarose-encapsulated islets for the prevention of complement activation ........ 19

INTRODUCTION ................................................................................................. 19

EXPERIMENTAL ................................................................................................. 21

Materials .............................................................................................................. 21

sCR1 purification ............................................................................................... 21

Chemical modification of sCR1 and agarose .................................................... 23

Protective effect of sCR1 immobilized on agarose hydrogel ......................... 24

Isolation and microencapsulation of islets ....................................................... 25

Inhibition of the complement-dependent destruction of islets by sCR1-immobilized on agarose ................................................................. 27

Statistical analysis ............................................................................................. 27

RESULTS ............................................................................................................ 28

Isolation and function of CHO sCR1 ............................................................. 28

Immobilization of sCR1 on agarose hydrogel ............................................... 30

Inhibitory effect of immobilized sCR1 on agarose ...................................... 32
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Islet morphology</td>
<td>33</td>
</tr>
<tr>
<td>Insulin release from islets</td>
<td>35</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>37</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>39</td>
</tr>
<tr>
<td>CHAPTER 2</td>
<td></td>
</tr>
<tr>
<td><strong>Xenotransplantation of sCR1 conjugated agarose microencapsulated islets</strong></td>
<td>45</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>45</td>
</tr>
<tr>
<td>EXPERIMENTAL</td>
<td>47</td>
</tr>
<tr>
<td>Animals</td>
<td>47</td>
</tr>
<tr>
<td>sCR1 purification</td>
<td>47</td>
</tr>
<tr>
<td>Islet isolation</td>
<td>48</td>
</tr>
<tr>
<td>Activation of agarose and microencapsulation of islets</td>
<td>48</td>
</tr>
<tr>
<td>Islet transplantation</td>
<td>49</td>
</tr>
<tr>
<td>Intraperitoneal glucose tolerance test (IPGTT)</td>
<td>50</td>
</tr>
<tr>
<td>Histochemical analysis</td>
<td>50</td>
</tr>
<tr>
<td>Static glucose stimulations of retrieved microcapsules</td>
<td>50</td>
</tr>
<tr>
<td>RESULTS</td>
<td>52</td>
</tr>
<tr>
<td>Immobilization of sCR1</td>
<td>52</td>
</tr>
<tr>
<td>Xenotransplantation of sCR1-agarose microcapsules</td>
<td>53</td>
</tr>
<tr>
<td>Intraperitoneal glucose tolerance test (IPGTT)</td>
<td>55</td>
</tr>
<tr>
<td>Graft retrieval and immunohistochemical examination</td>
<td>56</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>59</td>
</tr>
</tbody>
</table>
CHAPTER 3

Immobilization of soluble complement receptor 1 on islets .......................... 67

INTRODUCTION ............................................................................................... 67

EXPERIMENTAL ............................................................................................. 69

Synthesis of Mal-PEG-DPPE ......................................................................... 69

Purification of sCR1 ....................................................................................... 69

Immobilization of sCR1 on the islet surface using Mal-PEG-lipid ............... 70

Immunostaining of sCR1 on islets ................................................................. 72

Surface plasmon resonance (SPR) monitoring of sCR1 binding to Mal-PEG-
DPPE ............................................................................................................. 72

Protection of islets from xenoreactive antibodies and complement .......... 73

Glucose-stimulated insulin secretion from islets ................................. 74

Statistical analysis ....................................................................................... 74

RESULTS ......................................................................................................... 75

Protective effect of sCR1 ............................................................................. 75

SPR monitoring of sCR1 binding to Mal-PEG-DPPE .................................. 76

Immobilization of sCR1 on the islet surface ............................................. 77

Protective effect of sCR1 immobilized on islet surface ....................... 79

DISCUSSION ................................................................................................ 83

REFERENCES .............................................................................................. 85
CHAPTER 4

Layer-by-layer co-immobilization of soluble complement receptor 1 and heparin on islets ............................................................................................................. 89

INTRODUCTION ......................................................................................... 89

EXPERIMENTAL ....................................................................................... 91

Purification and thiolation of sCR1 ........................................................... 91

Surface plasmon resonance (SPR) monitoring of sCR1-heparin interactions ... 92

Immobilization of sCR1 and heparin on the islet surface ......................... 92

Observation of sCR1 and heparin immobilized on islet surfaces .............. 94

Anti-thrombin activity of immobilized sCR1-heparin ............................... 94

Protective effect of sCR1 from complement-mediated cytotoxicity .......... 95

Insulin secretion by modified islets ......................................................... 96

RESULTS .................................................................................................... 97

SPR monitoring of sCR1-heparin interactions ......................................... 97

Immobilization of sCR1 and heparin on islet surfaces ............................ 98

Anti-thrombin activity of immobilized heparin ....................................... 100

Protective effect of immobilized sCR1 .................................................. 101

Glucose-stimulated insulin secretion ...................................................... 104

DISCUSSION ............................................................................................ 106

REFERENCES ........................................................................................... 108

CHAPTER 5

Microencapsulation of islets with living cells using polyDNA-PEG-lipid conjugate
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CBB</td>
<td>Coomassie Brilliant Blue</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese Hamster Ovary</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPPE</td>
<td>1,2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's Buffered Salt Solution</td>
</tr>
<tr>
<td>HE</td>
<td>Hematoxylin and eosin stain</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>IBMIR</td>
<td>Instant blood mediated inflammatory reaction</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>KRB</td>
<td>Kreg-Ringer buffer</td>
</tr>
<tr>
<td>LAMs</td>
<td>Liposome-encapsulated agarose microbeads</td>
</tr>
<tr>
<td>LBL</td>
<td>Layer-by-layer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>Mal</td>
<td>Maleimide</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
</tr>
<tr>
<td>MTX</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>Mw</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NHS</td>
<td>Normal human serum</td>
</tr>
<tr>
<td>NHS-PEG-Mal</td>
<td>$\alpha$-N-Hydroxysuccinimidyl-$\omega$-maleimidyl poly(ethylene glycol)</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly(L-lysine)</td>
</tr>
<tr>
<td>PMPI</td>
<td>N-(p-maleimidophenyl) isocyanate</td>
</tr>
<tr>
<td>PolyA</td>
<td>Polyadenine</td>
</tr>
<tr>
<td>PolyT</td>
<td>Polythymine</td>
</tr>
<tr>
<td>RS</td>
<td>Rabbit serum</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SAM</td>
<td>Self-assembly monolayer</td>
</tr>
<tr>
<td>sCR1</td>
<td>Soluble human complement receptor 1</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>TMRE</td>
<td>Tetramethylrhodamine ethyl ester, and perchlorate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>U</td>
<td>Unit</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
</tbody>
</table>
GENERAL INTRODUCTION

Insulin-dependent diabetes mellitus (IDDM) type 1 is an autoimmune disease caused by the destruction of insulin-producing pancreatic β-cells [1-3]. As a consequence, there is insufficient insulin to regulate blood glucose level, thus resulting in hyperglycemia. Currently, treatment methods for this disease include insulin administration, whole pancreas transplantation, and islet transplantation [4]. Despite widely used as standard therapy, insulin administration results in poor control of blood glucose level leading to many complications such as neuropathy, nephropathy, heart diseases [5-7]. Whole pancreas transplantation required lifelong immunosuppressive therapy and major surgical procedure which usually associates with risks of complications [8].

Pancreatic islets are clusters of mainly insulin producing β cells accounting for about 1-2% of the pancreas [9]. As compared to whole organ transplant, islet transplantation is much simpler procedure. In clinical practice, islets are infused percutaneously into the patient’s liver through portal venous circulation [10]. With current developed technologies, islet transplantation can be conducted in a non-invasive manner using radiographic approach [11,12]. In addition, the separation of islets from pancreas allowed the application of many biotechnologies to improve cells properties and survival [13-18].
With the success of Edmonton protocol, islet transplantation becomes a promising alternative to treat type 1 diabetes mellitus [19-21]. However, there are still many issues needed to be addressed such as insufficient tissue supply, low efficacy of islet isolation, revascularization and immunorejection of transplanted islets [22-25]. Shortage of donor tissue has always been the major obstacle in organ and cell transplantation. Many technologies have been developed to solve this problem. Stem cells [26-28] and xenogenic tissues [29-31] are currently intensively studied as alternative sources for cell transplantation therapy. However, while stem cell technologies are still in developing stage; xenogenic transplantation has severe immunological challenges [32,33].

Immunorejection is the main reason for graft dysfunction in both allogenic and xenogenic islet transplantation [34]. This process involves both innate immune response through instant blood mediated inflammatory reaction (IBMIR) and the adaptive immune response. IBMIR is the major cause for the marked destruction (>50%) of islet mass early post-transplantation [35-37]. The two major processes in IBMIR are coagulation and complement cascade [38]. Coagulation cascade is triggered by several factors expressed on islet surface such as tissue factor (TF), collagen residues and MCP-1 [39-41]. Exposure of islets to fresh blood activates thrombotic reaction characterized by the activation of platelets inducing the formation of clot surrounding islets and the recruitment of neutrophils and macrophages which involve in the destruction of islets.

On the other hand, complement cascades could be activated in antibodies dependent or independent manner via three pathways namely classical pathway, alternative pathway and lectin pathway [42,43]. The activation of complement results in the recruitment of inflammatory cells, and stimulation of adaptive immune responses. In
addition, the assembly of the late complement components into a membrane attack complex (MAC) could penetrate the plasma membrane and induce cell death. Antibodies-mediated complement cytotoxicity is especially severe in the case of xenotransplantation since most human recipients have xenoreactive antibodies against animal tissues [44]. Xenoreactive antibodies and complement play a major role in the hyperacute rejection [45]. Therefore, it is crucially important to control complement activation in both of alloislet and xenoislet transplantation.

Several attempts have demonstrated that blocking coagulation and complement cascade could significantly improve graft survival and reduce islets mass necessary to reverse hyperglycemia [44,35,36]. The use of complement inhibitors such as hDAF [46], factor H [47-49], sCR1 [50-52], cobra venom factor [53,54] or anti-coagulants such as heparin [38,44], thrombomodulin [55,56], activated protein C [57,58] along with islet transplantation could markedly attenuate IBMIR and improve graft survival.

Among complement inhibitors, sCR1 attracts much attention since it is able to inhibit complement activation in both the classical and alternative pathways [59]. sCR1 has the ability to bind complement C3b/C4b, controls the dissociation of C3 and C5 convertase, and also serves as a co-factor for the proteolytic cleavage of C3b/C4b by factor I. It has been reported that sCR1 can prolong the survival of xenografts in a dose-dependent manner in several animal models and pre-clinical trials [60-65]. However, it is difficult to control the efficacy in the local microenvironment of the graft with systemic administration [49]. In addition, over treatment may affect the systemic immune responses or result in side effects and complications.
As an alternative approach, local treatment could be achieved via islet surface modification [66]. In this approach, islets are encapsulated in a very thin confomal membrane which protects islets from the immunogenic reactions. The methods to achieve this include covalent conjugation to amino groups of cell surface proteins, incorporation of amphiphilic polymers into the lipid bilayer membrane of cells by hydrophobic interaction and electrostatic interaction. The immobilization of poly(ethylene glycol) (PEG) [67-70], heparin [71-73] and functional proteins such as urokinase [74], thrombomodulin [75,76] has been reported to effectively prevent IBMIR.

Another method to protect transplanted cells from immune systems is via immuno-isolation by biocompatible hydrogels such as alginate [77-79], agarose [80-83], poly (ethylene glycol) (PEG) [84], polyvinyl alcohol (PVA) [85-86]. In these systems, islets are either micro- or macro-encapsulated by a semipermeable membrane which isolates islets from the immune system while still allow the transports of low molecular molecules such as oxygen, glucose, insulin and nutrients. These cellular constructs are called bioartificial pancreas. Our group previously developed a microcapsule-type bioartificial pancreas using agarose hydrogel and demonstrated its efficacy in allotransplantation models [80]. However, the agarose hydrogel is not sufficient to protect xenogeneic islets from rejection due to the diffusion of antibodies and complement into the membrane leading to the destruction of transplanted cells [87]. We have demonstrated that when the agarose network was rendered denser by increasing the concentration of agarose, graft survival was correspondingly prolonged because the diffusion of antibodies and complement components was further restricted [88]. In addition, the combination of agarose with complement consumption agent such as poly
(styrene sulfonic acid) (PSSa) also contributes to protection of xenogenic islets from humoral immunity [81]. However, protective effect of microcapsule containing PSSa is gradually lost when the adsorption of complement proteins is saturated.

In the present thesis, we aimed at developing the strategies to further enhance the protection of islets from the immune system using sCR1, heparin or living cells in order to reduce the necessary number of islets required and prolonged graft survival in both allo- and xeno-transplantation without use of immunosuppressive drugs. This thesis comprised of five chapters.

In Chapter 1, we aimed to develop a bioartificial pancreas that can realize xeno-islet transplantation. The islets were encapsulated in agarose microbeads carrying the soluble domain of human complement receptor 1 (sCR1), which is an effective inhibitor of the classical and alternative complement activation pathways. The conjugation ability of sCR1 onto activated agarose as well as viability and functionality of islets was determined. The protective effect of sCR1-agarose on xenoislets against antibody-complement-dependent destruction was examined by incubating the microencapsulated islets in rabbit serum.

Chapter 2 proceeds with in vivo evaluation of sCR1 conjugated agarose microencapsulated islets in a xenotransplantation model using streptozotocin (STZ) induced diabetic mice. Three groups of rats islets including naked islets, agarose microencapsulated islets and sCR1-agarose microencapsulated islets were transplanted into intraperitoneal cavity of diabetic mice. No immunosuppression was used. Graft function was assessed by following daily non-fasting blood glucose level and body weight of the recipients. Intraperitoneal glucose tolerance test (IPGTT) was conducted to
evaluate the glucose clearance ability of transplanted islets at designated periods post-transplantation. Histology and immunocytochemistry were also carried out to examine graft survival.

Chapter 3 describes our study on the surface modification of islet with human soluble complement receptor 1 (sCR1) to prevent complement activation during allo-islet and xeno-islet transplantation. In this chapter, sCR1 was immobilized on the islet cell surface through poly(ethylene glycol)-conjugated phospholipid (PEG-lipid) without the loss of islet viability and insulin secretion ability. We have demonstrated that sCR1 on islet surface could effectively inhibits complement activation and protects islets against attack by xenoreactive antibodies and complement.

In Chapter 4, human soluble complement receptor 1 (sCR1) and heparin were co-immobilized onto the surfaces of islet cells in layer-by-layer manner. sCR1 molecules carrying thiol groups were immobilized through maleimide-poly(ethylene glycol)-phospholipids anchored in the lipid bilayers of islet cells. Heparin was immobilized on the sCR1 layer via the affinity between sCR1 and heparin, and additional layers of sCR1 and heparin were formed layer-by-layer. The effects of these layers to prevent the activation of complement and coagulation cascades were then carefully examined in vitro.

Chapter 5 describes a new technique to microencapsulate islets with living cells using an amphiphilic poly(ethylene glycol)-conjugated phospholipid derivative (PEG-lipid) and DNA hybridization. PolyA and polyT were introduced onto the surfaces of the islets and HEK 293 cells, respectively, using amphiphilic PEG-lipid derivatives. PolyA20 modified HEK cells were immobilized onto the islet surface where polyT20-PEG-lipid
was incorporated. The behaviors of cells on islet surface as well as islet viability and insulin secreting function was carefully examined.
REFERENCES


CHAPTER 1

Immobilization of the soluble domain of human complement receptor 1 on agarose-encapsulated islets for the prevention of complement activation

INTRODUCTION

The transplantation of islets of Langerhans has been successfully applied to the treatment of patients with insulin-dependent diabetes mellitus (type 1 diabetes). However, several obstacles still remain, such as a shortage of human donors, low efficacy of islet isolation, and side effects of immunosuppressive drugs, among which the shortage of human donors is the most difficult to overcome. Recently, Hering et al. [1] reported that normoglycemia can be maintained for more than 100 days in STZ-induced diabetic cynomolgus macaques after intraportal transplantation of adult porcine islets. The study suggested opening up the use of pig islet xenograft transplantation to human patients. However, rationalizing the use of a large dose of immuno-suppressive drugs, which are required to control graft rejection in xenotransplantation, is difficult. The side effects of immunosuppressive therapy are expected to exceed the merits of islet transplantation.
The transplantation of islets enclosed in a semi-permeable membrane as a bioartificial pancreas has been studied as a method of islet transplantation free from immunosuppressive therapy. Islets can survive and control glucose metabolism for a long period of time in a host without immunosuppressive therapy because islets are isolated from the host immune system by a semi-permeable membrane. Various types of bioartificial pancreas have been proposed and developed, including islets microencapsulated within an alginate/poly(L-lysine) polyion complex membrane [2] or agarose hydrogel [3], or macroencapsulated by porous membranes [4-6] or living cells [7,8]. Our group previously developed a microcapsule-type bioartificial pancreas using agarose hydrogel and demonstrated its efficacy in allotransplantation models [3, 9-11]. However, the agarose hydrogel is not sufficient to protect xenogeneic islets from rejection. A combination of islet microencapsulation and mild immunosuppressive therapy could achieve xeno-islet transplantation [12].

In the present study, agarose hydrogel carrying a complement regulatory protein, the soluble form of complement-receptor type 1 (sCR1-agarose), was prepared and applied to encapsulate rat islets. Complement receptor type 1 (CR1) has been reported to be an effective inhibitor of the classical and alternative complement activation pathways [13-20]. The protective effect of sCR1-agarose on xeno-islets against antibody-complement-dependent destruction was examined by incubating the microencapsulated islets in rabbit serum.
EXPERIMENTAL

Materials

A Chinese hamster ovary (CHO35.6) cell line expressing sCR1 (CRL-10052™) [16] was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Alexa 488-labeled goat anti-mouse antibody and mouse anti-human sCR1 monoclonal antibody (J3D3) were purchased from Invitrogen (Carlsbad, CA, USA) and Beckman Coulter (CA, USA), respectively. 2-Iminothiolane hydrochloride (Traut’s reagent) was purchased from MP Biomedicals (Illkirch, France). The N-(p-maleimidophenyl) isocyanate (PMPI) was purchased from Thermo Fisher Scientific (Rockford, IL., USA). Agarose (Taiyo Agarose, AG LT-600) was obtained from Shimizu shokuhin KK (Shimizu, Japan), and paraffin oil (107162) was purchased from Merck (NJ, USA). Enzyme-linked immunosorbent assay (ELISA) kits for the insulin assay and CH50 autokit were purchased from Shibayagi (Gunma, Japan) and Wako Pure Chemical (Osaka, Japan), respectively. Collagenase was purchased from Nitta Gelatin (Osaka, Japan). The sources of other chemicals and culture media are listed in the supplemental information.

sCR1 purification

Human sCR1 was purified from the culture medium of CHO cells expressing sCR1 as previously described [21]. Briefly, the cells were first cultured in α-MEM medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 500 nM methotrexate (MTX). After the cells reached confluence,
they were washed three times with phosphate buffered saline (PBS) and cultured in serum-free ASF104 medium (Ajinomoto Co. Inc., Tokyo, Japan) supplemented with 500 nM MTX for an additional 2 days. The culture medium was collected and centrifuged at 1000 rpm for 5 min, the supernatant filtered through a membrane filter (φ:0.45 μm), and stored at 4°C before purification.

sCR1 was collected from the CHO culture medium by affinity column chromatography using a heparin sepharose 6B column. A 500 ml sample of the medium was loaded onto the heparin column and contaminants washed out using a 10 mM NaH₂PO₄ (pH 7.0) buffer solution. Bound sCR1 was eluted with an elution buffer composed of 10 mM NaH₂PO₄ and 1 M NaCl (pH 7.0). The protein concentration of the eluent was determined using a micro BCA protein assay kit. The collected sCR1 was subjected to SDS-PAGE analysis with coomassie brilliant blue (CBB) staining. The sCR1 solution was diluted with PBS and divided into 100 μl aliquots in 50 tubes and stored at -20°C until assayed. The sCR1 activity in the solution was evaluated with the CH50 assay using the CH50 autokit according to the manufacturer’s instructions and Yamamoto et al. with slight modifications [22]. Briefly, 250 μl hapten-liposome solution was added to a mixture of 9 μl of normal human serum (NHS) and 1 μl sCR1 solution (25 to 300 μg/ml) in PBS. The solution was incubated at 37°C. After 5 min, 125 μl of an antibody–substrate mixture was added to the solution. After incubating at 37°C for 4.6 min, absorbance was measured at 340 nm. The complement activation level (CH50 value) of the NHS-sCR1 mixture was determined from the absorbance using a standard curve. In the case of rabbit serum, a serum-sCR1 mixture was prepared by mixing 27 μl rabbit serum and 3 μl sCR1 solution in PBS (25 to 300 μg/ml). The CH50 assay was conducted as described for NHS.
Chemical modification of sCR1 and agarose

sCR1 was immobilized on the agarose hydrogel as shown in Scheme 1. First, sCR1 was thiolated using Traut’s reagent by mixing 1 ml of 2 mg/ml sCR1 solution in PBS with 10, 20, or 50 μl of 10 mg/ml Traut’s reagent solution in PBS by agitating for 1 h at room temperature. Thiolated sCR1 (sCR1-SH) was purified using a sephadex G25 column (GE Healthcare, Buckinghamshire, UK). The number of thiol groups introduced to the sCR1 molecule was determined using Ellman’s assay. The complement inhibition function of sCR1 before and after thiolation was determined by the CH50 assay.

Scheme 1. Schematic illustration of the immobilization of sCR1 on agarose. (a) Thiolation of sCR1 by Traut’s reagent. (b) Activation of agarose by PMPI. (c) Conjugation of sCR1-SH with the activated agarose.
The hydroxyl groups of agarose were modified to maleimide groups using PMPI as follows. PMPI (2 to 5 mg) was added to an agarose solution in dry DMSO (100 mg agarose in 5 ml DMSO) and stirred at room temperature under dark condition overnight. The modified agarose (Mal-agarose) was collected by pouring the reaction mixture into acetone. After decanting, the precipitated product was washed with acetone twice and dried under reduced pressure.

**Protective effect of sCR1 immobilized on agarose hydrogel**

Hapten-liposomes encapsulated in 2.5% agarose microbeads (LAMs) were utilized to examine the protective effect of sCR1 immobilized on agarose. Mal-agarose (150 mg) was dissolved in 3 ml PBS in a glass centrifugal tube using a microwave oven to prepare a 5% agarose solution. The agarose solution was left at 40°C for 7 min. The hapten-liposome suspension, which was supplied as a component of the CH50 assay kit and kept at 40°C, was added to an equal volume (3 ml) of the 5% agarose solution and mixed well. Pre-warmed liquid paraffin (15 ml, 40°C) was added to the glass tube, and the tube was vigorously agitation on ice for 3 min to induce gelation of the agarose microdroplets. Cold PBS (15 ml) was added to the glass tube and the suspension was centrifuged at 2000 rpm for 5 min at 4°C. The paraffin oil and supernatant were removed and the microbeads washed twice with PBS. The volume of the microbead suspension was adjusted to 500 μl after removing the supernatant. For immobilization of sCR1 on the agarose microbeads, 800 μg of sCR1-SH in 1 ml PBS was added to 500 μl of the microencapsulated liposome suspension, and the reaction mixture was incubated at 37°C for 2 h with gentle shaking every 30 min. After 2 hours, the microbeads were suspended
in PBS and centrifuged at 2000 rpm for 5 min at 4°C. This procedure was repeated 5 times to remove free sCR1-SH. As a control, a cysteine solution (1 mM), instead of the sCR1 solution, was added to agarose solution. The LAMs treated with or without sCR1 were stored in 10 ml PBS overnight to remove unreacted sCR1 and cysteine.

The CH50 assay using the CH50 autokit was modified to evaluate the inhibitory effect of sCR1 immobilized on agarose microbeads on complement activation. The sCR1-immobilized LAMs (200 μl) were mixed with 1 ml of 5-times diluted NHS in veronal buffer and was incubated at 37°C for 5 min. Then, 125 μl of the substrate solution was added to the mixture and kept at 37°C. Two hundred microliters of supernatant was collected at 25, 45, and 65 min during the incubation and the absorbance at 340 nm determined. The percentage of lysis in the LAMs was calculated from the absorbance value of completely lysed LAMs, which were incubated for 3 h in serum.

The amount of sCR1 immobilized to the agarose microbeads was determined by the micro BCA assay using microbeads without encapsulation of the hapten-liposome.

**Isolation and microencapsulation of islets**

Islets were isolated from male, 8-week-old ACI/N rats by the ductal injection of a collagenase solution [23]. Briefly, a rat was anesthetized by intraperitoneal injection of Nembutal (pentobarbital, 75 mg/kg). Approximately 10 ml of collagenase solution (0.5 mg/ml) was carefully injected through the common bile duct to the pancreas to distend the tissue. The pancreas was removed and kept at 37°C for 19 min to digest the pancreatic tissue. RPMI-1640 (5 ml) containing 10% FBS was added to the sample and the tissue was pipetted vigorously to release islets from the exocrine tissues. The
disintegrated tissue was washed twice with HBSS and the supernatant removed after centrifugation. The tissue was suspended in 5 m Ficoll/Conray solution (density: 1.1 g/ml) and transferred to a test tube. Ficoll/Conray solutions with a density of 1.075 g/ml and 1.050 g/ml (3 ml and 2 ml, respectively) were sequentially layered on the suspension to make a discontinuous density gradient. The test tube was centrifuged at 2500 rpm for 8 min. Islets were collected at the interface between the 1.075 g/ml and 1.050 g/ml layers. After washing the islets, they were transferred to culture medium (RPMI-1640) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin and cultured for 2 days before encapsulation with agarose microbeads.

Islets were encapsulated in sCR1-agarose microbeads using the same method as hapten-liposomes detailed above. Microencapsulated islets were cultured in RPMI1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO₂. The sCR1 immobilized in microbeads was visualized by immunostaining. After 2 days of culture, microbeads containing islets were kept in culture medium containing anti-human sCR1 monoclonal antibody (1 μg/ml, J3D3 mouse antibody) for 6 h at 37°C. The islets were washed and incubated in antibody-free medium for 1 day to complete remove unbound antibodies. The sCR1 in the microbeads was visualized by incubation in culture medium containing Alexa 488-labeled anti-mouse antibody (1 μg/ml, goat) for 6 h at 37°C, and then the Alexa 488-labeled antibody was extensively removed by culturing the microencapsulated islets in antibody-free medium for 24 h. The agarose microbeads were observed by confocal laser scanning microscopy (Fluoview, FV500, Olympus Optical Co. Ltd., Tokyo, Japan).
The ability of the microencapsulated islets to release insulin was examined by the static glucose-stimulated test [24]. Briefly, 50 microencapsulated islets were sequentially incubated in Krebs-Ringer solutions with glucose concentrations of 0.1 g/dl, 0.3 g/dl, and 0.1 g/dl for 1 h each at 37°C. At the end of each incubation, supernatants were collected and stored at -20°C until ELISA analysis of insulin concentrations.

**Inhibition of the complement-dependent destruction of islets by sCR1 immobilized on agarose**

Rabbit serum was used to examine the sCR1 protection of rat islets from complement-dependent destruction. Twenty naked islets and 20 islets encapsulated in agarose microbeads with or without sCR1 were cultured in 100% normal rabbit serum at 37°C. Serum was collected every 24 h and replaced by fresh serum after three washes with HBSS. The insulin contents of the collected sera were determined by ELISA. Islet morphology was observed by phase contrast microscopy (IX71, Olympus Optical Co. Ltd., Tokyo, Japan).

**Statistical analysis**

Comparisons between two groups were performed using the Student’s t-test. \( P < 0.05 \) was considered significant. All statistical calculations were performed using the software JMP ver.5.1.1.
RESULTS

Isolation and function of CHO sCR1

The SDS-PAGE of fresh ASF104 medium, conditioned CHO cell medium, and purified sCR1 is shown in Fig. 1A. A band appeared in the conditioned medium at roughly 250 kDa was assigned to sCR1 (Mw: 220 kDa). Bands at 80 kDa in the fresh and conditioned medium were assigned to transferin. After sCR1 was purified using a heparin column, most contaminants were removed (Fig. 1A, line c). Approximately 10-20 μg of sCR1 was isolated from 1 ml of culture medium.

The inhibitory effect of sCR1 on complement activation was determined with the CH50 assay. The CH50 value decreased with increasing sCR1 concentrations (Fig. 1B), indicating that sCR1 isolated from the conditioned CHO cell medium can effectively inhibit complement activation.

In xenotransplantation, complement regulatory proteins, such as decay accelerating factors and CD46, are used to impair the assembly of the membrane attack complex. In some donor and recipient combinations, however, the inhibitory properties of these molecules are not effective due to species restriction [25]. We examined the inhibitory effects of human sCR1 on complement activation in rabbit serum. With the addition of sCR1 to the serum, the increased absorbance at 340 nm remained low (Fig. 1C). This finding indicates that sCR1 can also effectively inhibit complement activation in rabbit serum.
Figure 1. Analysis of sCR1 isolated from CHO35.6 cell culture medium. (A) SDS-PAGE of sCR1 before and after purification using a heparin column. a: ASF104 medium, b: CHO culture supernatant, c: fraction from the culture medium collected through the heparin column. (B) CH50 values of 90% normal human serum supplemented with certain amounts of sCR1. (C) Inhibitory effect of sCR1 in 90% rabbit serum.
CHAPTER 1 Immobilization of sCR1 on agarose-encapsulated islets

Immobilization of sCR1 on agarose hydrogel

The number of thiol groups introduced on sCR1 increased with increasing Traut’s reagent concentration (Fig. 2). The CH50 value of NHS was 38.9 U/ml. When naïve sCR1 was added to NHS at 2 μg/ml, the CH50 value decreased to 24.6 U/ml. When sCR1-SH carrying 4.6, 7.6, or 10.8 thiol groups per molecule were added to NHS at 0.26 μg/ml, the CH50 value decreased to 26.46, 26.18, and 28.09 U/ml, respectively (Fig. 3). Although the inhibitory function of sCR1 tended to decrease with an increasing number of introduced thiol groups, it did not exert a deteriorative effect on sCR1 function. The CH50 value of sCR1 with 4.6 thiol groups per molecule was almost the same as that of naïve sCR1. sCR1 carrying 4.6 thiol groups was used in the following experiments.

![Figure 2. Number of thiol groups introduced per sCR1 molecule compared to the molar ratio of Traut’s reagent to sCR1.](image-url)
CHAPTER 1 Immobilization of sCR1 on agarose-encapsulated islets

![Bar chart showing CH50 values for different thiol groups per sCR1 molecules.]

**Figure 3.** Effect of thiolation on sCR1 activity. CH50 values are shown for 90% normal human serum and 9 μl serum supplemented with 1 μl naïve sCR1 or thiolated sCR1 (0.26 μg/ml) as indicated.

![Graph showing the amount of immobilized sCR1 on agarose activated with varying amounts of PMPI.]

**Figure 4.** Amount of immobilized sCR1 on agarose activated with varying amounts of PMPI. sCR1-SH carrying 4.6 thiol groups/sCR1 was used.

Maleimide groups were introduced to agarose by PMPI and sCR1-SH was covalently immobilized on agarose through the thiol-maleimide reaction. Microbeads made of agarose modified with PMPI were immersed in a sCR1-SH solution to
immobilize sCR1 to the agarose microbeads. The amount of sCR1 in the sCR1-agarose conjugates was determined using the micro BCA method (Fig. 4).

**Inhibitory effect of immobilized sCR1 on agarose**

Hapten-liposomes were enclosed in 2.5% sCR1-agarose microbeads and then incubated in NHS to examine the inhibitory function of immobilized sCR1. When the complement system is activated on the hapten-liposome, the enzyme enclosed in the liposome is released into the solution and subsequently releases chromophore from the substrate, increasing the absorbance of the supernatant at 340 nm. The percentage of damaged liposome in NHS was evaluated from the changes in absorbance at 340 nm (Fig. 5).

**Figure 5.** Inhibitory effect of sCR1 on the complement-dependent lysis of hapten-liposome. Dark circles indicate agarose microbeads, white circles indicate sCR1-agarose microbeads, and squares indicate agarose microbeads suspended in a medium supplemented with sCR1 (50 μg/ml). The average diameter of the agarose microbeads was 200 μm.
5). Hapten-liposomes enclosed in agarose microbeads without sCR1 were also incubated in NHS and NHS supplemented with 50 μg/ml sCR1. Although the absorbance of the supernatants increased with time in all three conditions, the absorbance for sCR1-agarose microbeads increased much more slowly than those without immobilized sCR1. Immobilized sCR1 effectively inhibited the complement activation, comparable to sCR1 in NHS.

**Islet morphology**

Islets were encapsulated in microbeads made of agarose modified with PMPI. The microencapsulated islets were immersed in a solution of sCR1-SH immobilized to aCR1 in the agarose microbeads. To examine the immobilization of sCR1 in agarose microbeads, the microbeads were immuno-stained using anti-sCR1 antibody. Clear green fluorescence was observed on the agarose microbeads (Fig. 6B). However, no fluorescence was observed for agarose not activated with PMPI. These results indicate that sCR1 was covalently immobilized on the agarose hydrogel through the thiol/maleimide reaction. Rat-rabbit is a discordant combination [26], as antibodies that react with rat tissue exist in rabbit serum. When naked rat islets are exposed to fresh rabbit serum, the antibodies form antigen-antibody complexes on islets and the immune complexes activate the complement system through the classic pathway, resulting in the destruction of islet cells. The protective effect of sCR1 immobilized on agarose in regards to the antibody-complement dependent destruction of islets was examined by incubating microencapsulated islets in rabbit serum. Naked rat islets were rapidly destroyed over a
few days in rabbit serum, whereas no morphological change was observed in islets encapsulated in sCR1-agarose microbeads (Fig. 6A). The sCR1-agarose microbeads effectively protected the islets. Figure 6 also includes images of islets encapsulated in 5% agarose microbeads without sCR1 immobilization. Although some cells located at the periphery were swollen, the islets kept their intact round shape even after 4 days in rabbit serum.

**Figure 6.** Phase contrast microscope images of microencapsulated islets (A) and Immunofluorescence staining of sCR1 using anti-sCR1 antibody (J3D3 primary mAb) and Alexa 488 labeled anti-mouse antibody (B). (A) Protection effect of sCR1-agarose microbeads. Islets were incubated in 100% normal rabbit serum for the indicated time: a: Islet sCR1-agarose microbeads, b: islets in agarose microbeads without sCR1, c: naked islets. Scale bars = 200 μm. (B) a: sCR1-agarose microbead, b: agarose microbead without sCR1. Scale bars = 100 μm.
serum. Agarose microbeads alone have some protective effect against the antibody-complement-dependent destruction of islets.

**Insulin release from islets**

The glucose stimulation test was carried out to determine the effects of microencapsulation and sCR1 immobilization on islet function (Fig. 7). The release of insulin from islets in microbeads with or without sCR1 at high glucose concentrations was about 20% less than that of naked islets, with no difference observed between the two groups of islets in microbeads. Although the microencapsulation procedure exerted some deteriorative effects on islet function, islets in microbeads carrying sCR1 still maintained the ability to release insulin and control the amount released in response to the glucose concentration.

![Glucose stimulation tests of naked islets and microencapsulated islets (MIs) with or without sCR1.](image)

**Figure 7.** Glucose stimulation tests of naked islets and microencapsulated islets (MIs) with or without sCR1.
β-cells in islets contain insulin as granules. When islets are damaged in rabbit serum, insulin leaks from the damaged β-cells. The amount of insulin in rabbit serum was determined to quantitatively evaluate islet cell death (Fig. 8). From 20 naked islets cultured in rabbit serum, 551.1 ng insulin leaked during the first 24 h period and 239.4 ng insulin leaked during the second 24 h period. After two days, the amount of insulin released drastically decreased because most of the β-cells were destroyed during the initial two days. The amount of insulin released from the islets in microbeads was much less than that of naked islets over the course of two days in culture. The amount of insulin leaked from islets in microbeads carrying sCR1 was half that leaked from islets in microbeads without sCR1 during. These observations indicate that the agarose hydrogel effectively protects islets from antibody-complement-dependent cell damage and sCR1 immobilized on agarose exerted an additional protective effect.

![Graph](image)

**Figure 8.** The amount of insulin release was determined to measure β-cell lysis during islet incubation in rabbit serum for 4 days. ■: Naked islets, □: Islets in agarose microbeads without sCR1, ☉: Islets in sCR1-agarose microbeads.
CHAPTER 1 Immobilization of sCR1 on agarose-encapsulated islets

DISCUSSION

Our group has been examining agarose as a material to microencapsulate islets and demonstrated that agarose microbeads effectively protect allogeneic islets from the immune attack of recipient mice; thus, islet graft survival was realized without immunosuppressive therapy for a long period of time [3]. Though hamster islets enclosed in agarose microbeads cannot survive so long in recipient mice, we demonstrated that a combination of islet microencapsulation and B cell suppression by the administration of 15-deoxyspergualin can realize long normoglycemia in islet xenotransplantation between hamster and mouse [12]. However, the administration of 15-deoxyspergualin is not a good choice for diabetic patients when its side effects are taken into consideration.

In order to improve xenograft survival, some groups have examined the immobilization of bioactive substances to hydrogel macrocapsules [27-30]. An anti-inflammatory peptide was immobilized to functionalized hydrogel crosslinked by the thioester/thiol reaction [31]. In another study, anti-Fas mAb was conjugated to PEG hydrogel using photopolymerization [32]. These approaches have shown promising results for the down-regulation of inflammatory reactions and are expected to improve graft survival. However, the chemical reaction employed and necessary UV exposure are expected to exert deteriorative effects on cell viability and protein function. In addition, the activation of the complement system should be controlled for the success of xenogeneic islet transplantation because antibody-complement-dependent cytotoxicity plays a major role in xenograft rejection.
In this study, islets were enclosed in sCR1-agarose microbeads because sCR1 has the ability to bind complement C3b/C4b, controls the dissociation of C3 and C5 convertase, and also serves as a co-factor for the proteolytic cleavage of C3b/C4b by factor I. sCR1 is also able to inhibit complement activation in both the classical and alternative pathways, and it has been reported that sCR1 can prolong the survival of xenografts in a dose-dependent manner in several animal models and pre-clinical trials [13-20]. However, controlling the sCR1 level in the local microenvironment of the graft is difficult with systemic administration of sCR1 [33]. In this study, sCR1 was immobilized on agarose by the thiol/maleimide reaction and sCR1-agarose was used to microencapsulate islets. The local concentration of sCR1 surrounding the islets increased for the effective regulation of antibody-complement-dependent cytotoxicity. The advantage of the thiol/maleimide reaction is that agarose is modified with maleimide groups before microencapsulation of islets, and proteins carrying thiol groups can be immobilized onto the agarose microbeads containing living islets under physiological conditions.

When naked rat islets were cultured in rabbit serum, large amounts of insulin leaked from the damaged islets over the course of a few days incubation (Fig. 8). However, no damaged cells were seen on the islets enclosed by sCR1-agarose microbeads (Fig. 6). Low levels of insulin were detected in the rabbit serum, but this was not due to leakage of insulin from damaged β-cells, it was physiological insulin secretion by β-cells.
REFERENCES


CHAPTER 2

Xenotransplantation of soluble complement receptor 1 conjugated agarose microencapsulated islets

INTRODUCTION

Following the successes of Edmonton protocol, pancreatic islet transplantation has become the promising alternative for the treatment of type 1 diabetes mellitus. Although many improvements in the outcomes of islet transplantation have been reported [1-3], the challenge with the insufficient tissue supply is still remained. Stem cells [4-6] and xenogenic tissues [7-9] are currently intensively studied as alternative sources for cell transplantation therapy. However, while stem cell technologies are still in developing stage; xenogenic transplantation has severe immunological challenges [10,11]. Humoral immunity plays the major role in the destruction of xenogenic graft. Upon transplantation, a vast amount of xenoreactive antibodies existed as preformed antibodies in the host rapidly bind to the transplanted tissue and activate the innate immune system in which complement plays the central role. The activation of complement system amplifies the inflammatory responses by the generation of many potent anaphylatoxins [12,13]. The grafts are then destroyed either by the membrane attack complex which assembled at the terminal of complement activation process or by immune cells such as macrophages,
neutrophils which are recruited to the transplanted area [14]. Currently, immunosuppressive therapy is used to protect the graft from rejection [15]. Despite effective, it is not practical in clinical applications due to the potential side effects and complications [16].

Another method to protect transplanted cells from immune systems is via immuno-isolation by biocompatible hydrogels such as alginate [17-19], agarose [20-23], poly (ethylene glycol) (PEG) [24], polyvinyl alcohol (PVA) [25,26]. In these systems, islets are either micro- or macro-encapsulated by a semipermeable membrane which isolates islets from the immune system while still allows the transports of low molecular molecules such as oxygen, glucose, insulin and nutrients. However, although these systems received some successes in allotransplantation, poor outcomes have been reported in xenotransplantation due to the permeability of antibodies and complement leading to the destruction of transplanted cells [27-29].

In the previous study, we have developed a new method aiming to both physical isolation and complement inhibition [30]. In this method, islets were microencapsulated with agarose which is immobilized with soluble complement receptor 1, a potent complement inhibitor for both classical and alternative complement activation pathway [31-33]. We have demonstrated in vitro that this technique could inhibit complement activation and provide a better protection for islets against the destruction of xenoreactive antibodies and complement system in the serum as compared to non-treated microcapsules. Herein, we conduct in vivo studies to evaluate the performance of sCR1-conjugated agarose microencapsulated islets in a xenotransplantation model using streptozotocin (STZ) induced diabetic mice.
EXPERIMENTAL

Animals

Male ACI/NSlc rats, 8-week-old, and male C57BL/6 mice, 8-week-old (Shimizu Co., Japan) were used as donors and recipients, respectively. Diabetes was induced in C57BL/6 mice by a single intraperitoneal injection of streptozotocin (STZ) (210 mg/kg body weight in citrate buffer, pH 4.5) (mixed anomers, Nacalai Tesque, Kyoto, Japan)). Mice with blood glucose level exceeded 450 mg/dL in two consecutive measurements were used as diabetic recipients. All animal experiments were carried out according to the guidelines of The Kyoto University Animal Care Committee.

sCR1 purification

Human sCR1 was prepared using Chinese hamster ovary (CHO) cells expressing sCR1 (CRL-10052TM, ATCC; Manassas, VA, USA) as described previously [34]. Briefly, CHO cells were allowed to expand in α-MEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Equitech-Bio, Kerrville, TX, USA), 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen), and 500 nM methotrexate (Sigma-Aldrich, St. Louis, MO, USA). Medium was then changed to serum-free ASF104 medium (Ajinomoto Co. Inc., Tokyo, Japan) supplemented with 500 nM Methotrexate. Supernatants containing sCR1 were collected after 2 days culture, pooled together and stored at 4°C. sCR1 was purified from the supernatants by affinity chromatography using a HiTrap Heparin HP column (GE Healthcare, Buckinghamshire, UK), and the eluent
was further purified with a centrifugal filter device (Amicon Ultra -15, 100 kDa; Milipore Corporation, MA, USA). The sCR1 concentration was determined using a micro BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA).

For thiolation, 1 mL of 3 mg/mL sCR1 solution was mixed with 100 μL of 10 mg/mL Traut’s reagent in phosphate-buffered saline (PBS; MP Biomedicals, Illkirch, France). The solution was left at room temperature for 1 hour, after which thiolated sCR1 (sCR1-SH) was purified by a Sephadex G25 column (GE Healthcare). Ellman’s reagent (Nacalai Tesque, Kyoto, Japan) was used to determine the number of thiol groups per sCR1 molecule.

Islet isolation

Islets were isolated from ACI/N rats (male, 8-week-old, Shimizu Co., Japan) by the conventional collagenase digestion method as described previously [35]. Islets were purified from digested tissues by centrifugation using a discontinuous density gradient of Ficoll/Conray solutions. Islets were maintained in culture medium (RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin) for 2 days before microencapsulation.

Activation of agarose and microencapsulation of islets

The hydroxyl groups of agarose were modified to maleimide groups using N-(p-maleimidophenyl) isocyanate (PMPI) as described previously [30]. PMPI (9 mg) was added to an agarose solution (300 mg agarose in 15 ml dry DMSO) and stirred at room temperature overnight. The crude product was precipitated in acetone. After decanting,
the precipitate was washed with excessive amount of acetone five times and dried under reduced pressure. Mal-agarose was obtained as white powder (yield 80%).

For microencapsulation of islets, 150 mg agarose or Mal-agarose were dissolved in 3 ml PBS in a glass centrifugal tube using a microwave oven to prepare a 5% agarose solution. The agarose solution was left at 40°C for 7 min. Islets were washed with Hank's Buffered Salt Solution (HBSS) and islet suspension in minimum HBSS was added to agarose solution. 15 ml of pre-warmed liquid paraffin (Merck, NJ, USA) was added to the glass tube. The tube was briefly agitated to emulsify, then vigorously shaken on ice for 3 min to induce gelation of the agarose microdroplets. Cold HBSS (15 ml) was added to the glass tube and the suspension was centrifuged at 2000 rpm for 5 min at 4°C. The paraffin oil and supernatant were removed and the microbeads were washed with HBSS three times. For conjugation of sCR1, Mal-agarose microbeads were incubated in 5 ml thiolated sCR1 solution (500 μg/ml in KRB) for 3h at RT. sCR1-agarose microbeads were washed 3 times with culture medium and cultured for 1 day before transplantation.

**Islet transplantation**

Diabetic mice were anesthetized by inhalation of isofurane using a specialized instrument (400 Anesthesia Unit; Univentor, Malta). Agarose or sCR1-agarose microencapsulated islets (500-1000 islets) in 300-400 μl HBSS were injected into peritoneal cavity of diabetic mice through a small abdominal incision. The wound was closed with a 9 mm Autoclip® Applier (Becton Dickinson, NJ., USA). Blood samples were collected from tail vein of the recipient mice every 1 to 2 days (between 11:00 AM and 1:00 PM) to measure non-fasting blood glucose levels using a glucose sensor.
(DIAmeter-α glucocard; Arkray, Kyoto, Japan). Graft was considered dysfunction when two consecutive blood glucose levels exceeded 200 mg/dL.

**Intraperitoneal glucose tolerance test (IPGTT)**

Intraperitoneal glucose tolerance test (IPGTT) was carried out at 30th and 50th day postransplantation to evaluate the function of transplanted islets [36]. Briefly, mice were intraperitoneally injected with glucose solution (2 g glucose/kg body weight). Blood glucose levels were measured at 0, 15, 30, 60, 90, 120 min after inoculation. Normal and non-treated diabetic mice were used as controls.

**Histochemical analysis**

In some recipients receiving control microcapsules or sCR1-agarose microcapsules, grafts were retrieved after 31 days post-transplantation for histochemical examination using hematoxylin and eosin stain as described previously [36]. Immunohistochemistry was also carried out to examine the presence of insulin and sCR1 in the microcapsules using anti-insulin (rabbit polyclonal antibody, Thermo Scientific; Rockford, IL., USA) and anti-sCR1 (J3D3, Invitrogen, Carlsbad, CA, USA), respectively. To confirm whether the glucose level was controlled only by only transplanted islets, pancreas of the recipients were also dissected at the end of the study for histochemical analysis.

**Static glucose stimulations of retrieved microcapsules**

Microcapsules retrieved from peritoneal cavity of mice were subjected to glucose stimulation assay to evaluate the insulin response of islets [37]. Briefly, microcapules
were washed 3 times and incubated in Krebs-Ringer buffer (KRB) with 0.1 mg/ml, 0.3 mg/dL and 0.1 mg/dL glucose concentration, each for 2h at 37°C. Supernatant was collected after each incubation and subjected to ELISA assay (Shibayagi, Gunma, Japan) to determine insulin secretion from islets.
RESULTS

Immobilization of sCR1

Fig. 1 shows the immobilization of sCR1 on to agarose membrane of microencapsulated islet as described in Materials and methods. To observe the binding of sCR1 on agarose membrane, we conducted immunostaining of the microcapsules using anti-sCR1 (J3D3) and counterstained with Alexa488 labeled antibody. Clear fluorescence

Figure 1. Immobilization of sCR1 onto agarose microencapsulated islets. sCR1-agarose microbeads (A1-A2) and agarose microbeads (B1-B2) were immunostained with anti-sCR1 antibodies (J3D3) and counterstained with green fluorescent-labeled Alexa488 antibodies. (C) Phase contrast microscope image of sCR1-agarose microencapsulated islets after 2 weeks culture in RMPI 1640 supplemented with 10% FBS. Scale bar 200 μm.
was observed on agarose membrane of microcapsules when sCR1 was immobilized onto activated agarose. In contrast, no fluorescence was seen on control microcapsule prepared by normal agarose. These results indicated that sCR1 could be covalently conjugated onto agarose membrane using this technique. Fig. 1 also includes phase contrast photograph of islet enclosed in sCR1-agarose after cultured for 2 weeks. The intact morphology of islets indicated that there is no effect to islet viability after the conjugation procedure.

**Xenotransplantation of sCR1-agarose microcapsules**

To examine the protective effect of sCR1, we carried out the xenotransplantation of rats islets encapsulated in either sCR1-agarose microcapsules (sA-Mics) or in normal agarose microcapsules (Mics). Blood glucose level of all mice receiving 500 and 1000 microcapsules of both groups became normal (<200 mg/dL) within 1-3 days post-transplantation (Fig. 2). In the non-treated microcapsule group, normoglycemia was only maintained for a short period after which reversal to hyperglycemia was observed. The body weight of mice partially increases for 5 to 10 days and start to decrease after 1 to 2 weeks post-transplantation. Graft survival after transplanted 500 and 1000 Mics were 7.2 ± 2.3 days and 12.8 ± 4.2 days (Table 1). In contrast, when 500 and 1000 sA-Mics were transplanted, graft survival was significantly prolonged to 16.2 ± 2.5 days and more than 32 ± 10.7 days, respectively. 4 out of 7 mice transplanted with 1000 sA-Mics could maintain stable normoglycemia for more than 30 days (Fig. 2(E)). The body weight of mice receiving 1000 sA-Mics steadily increased for more than 50 days even after blood glucose became hyperglycemia again.
**Figure 2.** Assessment of graft survival after xenotransplantation of sCR1 agarose or non-treated agarose microencapsulated islets. 500 microcapsules (A and B) or 1000 microcapsules (C and D) from each group were injected into intraperitoneal cavity of
STZ-diabetic mice. Blood glucose level (A and C) and body weight (B and D) of the recipient mice were followed every 1 to 2 days post-transplantation. * indicated mice killed for histological examination. (E) Percentage of graft survival post-transplantation. Grafts were considered rejected when two consecutive blood glucose levels exceeded 200 mg/dL. M500 and M1000 indicated mice transplanted with 500 and 1000 non-treated agarose microencapsulated islets. SA500 and SA1000 indicated mice transplanted with 500 and 1000 sCR1-agarose microencapsulated islets.

### TABLE 1. Graft survival after xenotransplantation of sCR1-agarose microencapsulated islets

<table>
<thead>
<tr>
<th>Number of islets</th>
<th>Agarose microcapsule</th>
<th>sCR1-agarose microcapsule</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Graft survival (days)</td>
<td>Average</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>13, 4, 7, 5, 7</td>
<td>7.2 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>10, 20, 19, 15, 16, 17</td>
<td>16.2 ± 2.5</td>
</tr>
<tr>
<td>1000</td>
<td>14, 8, 12, 22, 8</td>
<td>12.8 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>20, 38, 20, 55, 24, 35</td>
<td>32 ± 10.7</td>
</tr>
</tbody>
</table>

#### Intraperitoneal glucose tolerance test (IPGTT)

At 30th and 50th day post-transplantation, glucose tolerance test was performed in the transplanted mice to evaluate the function of the grafts. Normal and non-treated diabetic mice were used as controls. Fig. 3 demonstrates blood glucose profiles of non-diabetic, diabetic mice and mice receiving 1000 Mics or 1000 sA-Mics at 30th and 50th day post-transplantation after intraperitoneal injection with glucose solution (2 g/kg body weight).

In all mice, after glucose loading, blood glucose level was rapidly increased. In mice receiving 1000 sA-Mics, a rapid reversal of blood glucose level was observed. No substantial difference in blood glucose profiles was observed between sA-Mics mice and non-diabetic mice. Blood glucose level of sA-Mics mice at 30th day tends to be slightly lower than at 50th post-transplantation. Similar glucose profiles were seen in control Mics
recipients (at 30th day) and in diabetic mice. After 120 min of glucose loading, no reversal of glucose level was observed in these mice.

Figure 3. Intraperitoneal glucose tolerance test on mice transplanted with 1000 sCR1-agarose microencapsulated islets at 30th day (dark circles) and at 50th day (dark triangles) post-transplantation. White circles indicated mice receiving 1000 non-treated agarose microencapsulated islets at 30th day post-transplantation. Non-diabetic (dark squares) and diabetic (white squares) were used as controls. Glucose solution (2g/kg body weight) was injected into peritoneal cavity of mice and blood glucose level was measured at 0, 15, 30, 60, 90, 120 min after inoculation.

Graft retrieval and immunohistochemical examination

Transplanted grafts were retrieved from recipient mice at 31st posttransplantation for histochemical examination. Fig. 4 shows the phase contrast microscopic images and sCR1 staining of grafts retrieved from mice receiving non-treated microcapsules and sCR1-agarose microcapsules. The microcapsules in both groups remained intact with very few broken capsules observed (Fig. 4 (A) and (B)). No infiltration of immune cells
was observed suggesting the destruction of islets was due to humoral immunity. Intact islets could be found in the experimental group indicated by robust expression of insulin but not in the non-treated microcapsule group (Fig. 5). To confirm whether blood glucose level was only controlled by the graft, during graft retrieval, pancreas was also dissected and subjected to H&E and insulin staining to examine the presence of viable \( \beta \) cells from the host (Fig. 5(C)). Very few insulin stained cells was observed in the native pancreas indicating that blood glucose level in the recipient mice was controlled only by

---

**Figure 4.** Phase contrast microscope images of agarose (A) and sCR1-agarose (B) microbeads retrieved at 31st day post-transplantation. Scale bars 200 µm. (C) Immunostaining of sCR1-agarose islets retrieved at 31st post-transplantation. microbead was stained with anti-sCR1 (FITC-J3D3) and observed under confocal microscope. Scale bars 100 µm. (D) Glucose stimulated insulin secretion test of sCR1-agarose islets retrieved at 31st post-transplantation.
transplanted islets. Immunostaining of retrieved microcapsules from experimental group at 31st day post-transplantation also revealed the presence of sCR1 indicated by green fluorescence on agarose membrane (Fig. 4(C)).

Figure 5. Histochemical examination of sCR1-agarose islets (A) and agarose microencapsulated islets (B) after 31 days xenotransplantation in STZ-diabetic mice. Scale bar 50 μm. Arrows indicated blood vessels formation in the vicinity of the grafts. (C) Histochemical examination of dissected pancreas of mice receiving sCR1-agarose islets at 31st post-transplantation. Scale bar 100 μm.

Glucose stimulation was also carried on retrieved islets to determine whether transplanted islets could retain insulin secretion function in response to glucose. As shown in Fig. 4(D), despite weaker than normal fresh isolated islets, the insulin response to glucose was still seen with stimulation index 1.99 ± 0.18.
DISCUSSION

To overcome the challenge with insufficiency of donor tissue, we aim to develop a method to realize xeno-islet transplantation. In previous study, we have successfully developed a technique to conjugate sCR1, a potent complement inhibitor, onto agarose membrane [30]. We have demonstrated *in vitro* that sCR1 immobilized in this manner still retains complement inhibitory function and protects islets against destruction over the course of serum incubation.

In this study we carried out *in vivo* studies to evaluate the performance of sCR1-conjugated agarose microencapsulated islets in xenotransplantation model using STZ-induce diabetic mice. Severe diabetic mice were transplanted with 500 or 1000 islets microencapsulated in either non-treated agarose or sCR1-agarose. Mice receiving either 500 or 1000 control microcapsules became normoglycemia for only 7.2 ± 2.3 days and 12.8 ± 4.2 days, respectively, after which reversal to hyperglycemia was seen (Fig. 2). This is consistent with previous results [27,28], since agarose network at this condition is permeable to antibodies and complement system. This is also indicated by the fact that sCR1 (Mw = 250 kDa), a larger molecule than antibodies (Mw = 150 kD) and most of complement components [38], could readily diffuse into agarose membrane during the immobilization procedure. The diffusion of antibodies and complement resulted in rapid destruction of islets, thus leading to graft dysfunction soon after transplantation. In mice receiving 500 or 1000 sCR1-agarose microcapsules, graft survival was markedly prolonged to 16.2 ± 2.5 days and more than 32 ± 10.7 days, respectively. IPGTT revealed
that sA-Mics grafts at 30th and 50th day post-transplantation could function to reverse hyperglycemia in very similar manner with non-diabetic mice (Fig. 3). In histochemical examination at 31st posttransplantation, viable islets with robust insulin expression could be found in mice receiving sCR1-agarose microcapsules but not in those transplanted with normal microcapsules (Fig. 4). These results indicated that sCR1 on agarose has exerted protective effect on islets against antibodies-mediated complement cytotoxicity and contributed to maintain normoglycemia in mice for a prolonged period without use of immunosuppressive therapy. Interestingly, immobilized sCR1 still existed after 1 month transplantation. The presence of sCR1 is expected to provide the continuous protection to islets by controlling complement activation in the vicinity of the grafts. Although mice receiving sCR1-agarose islets gradually became hypoglycemia, the dysfunction of graft may also be accounted to other factors such as revascularization or proinflammatory and proapoptotic cytokines (TNF-α, iNOS, MCP-1, ect …). Optimization of these parameters is expected to significantly contribute to the realization of xeno-islet transplantation.
REFERENCES


CHAPTER 3

Immobilization of soluble complement receptor 1 on islets

INTRODUCTION

Transplantation of pancreatic islets of Langerhans (islets) has been accepted as a promising method to treat insulin-dependent diabetes mellitus [1]. The success of the Edmonton protocol encourages further clinical trials of islet transplantation [2,3]. However, several pancreas donors are necessary to achieve insulin independence in one recipient [4], because more than 50% of islets are destroyed immediately after intraportal transplantation [5]. Early graft loss is reportedly caused by the instant blood-mediated inflammatory response (IBMIR) [6]. Exposure of islets to fresh blood activates the coagulation cascade and innate immune responses including complement activation, resulting in islet destruction by nonspecific inflammatory reactions. Suppression of those initial unfavorable reactions could rescue many islets, increasing the expectation that islet transplantation from one donor to one recipient may be achieved. Even when a protocol for successful islet transplantation from a single donor is established, donated tissue will likely be insufficient to cure a large number of diabetic patients. Porcine islets have been accepted as a promising alternative [7]. However, most human recipients have xenoreactive antibodies against porcine tissue. Xenoreactive antibodies and complement play a major role in the hyperacute rejection
It is crucially important to control complement activation in both of alloislet and xenoislet transplantation.

Complement receptor type 1 (CR1), which is a membrane glycoprotein expressed on the surface of various blood cells, kidney podocytes, and dendritic cells, is a potent inhibitor for both classical and alternative complement activation pathways. CR1 induces the dissociation of C3 and C5 convertases and acts as a cofactor for the proteolytic cleavage of C3b and C4b by factor I. The soluble domain of human CR1 (sCR1) has been prepared by gene transfection into Chinese Hamster Ovary (CHO) cells. It has been demonstrated that systemic administration of purified sCR1 can prolong graft survival in organ allotransplantation and xenotransplantation. A large amount of sCR1 is needed for its systemic administration, and systemic inhibition of the complement system may cause various unwanted side effects. Local control of complement activation is desired.

Our group has immobilized bioactive substances, such as urokinase and thrombomodulin, on islets using poly(ethylene glycol)-conjugated phospholipid (PEG-lipid) derivatives to control IBMIR. In this study, thiol groups were introduced onto sCR1 (sCR1-SH), which was then immobilized on rat islets using a PEG-lipid derivative carrying a maleimide end group. The sCR1-immobilized rat islets were incubated in rabbit serum to examine how well sCR1 protected islets from cell destruction by complement activation.
CHAPTER 3 Immobilization of sCR1 on islets

EXPERIMENTAL

Synthesis of Mal-PEG-DPPE

1,2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine (DPPE) (NOF Corporation, Tokyo, Japan) and α-N-Hydroxysuccinimidyl-ω-maleimidyl PEG (mal-PEG-NHS, Mw 5000) (Nektar Therapeutics, San Carlos, USA) were used to prepare maleimide-poly(ethylene glycol)-conjugated phospholipid derivative (Mal-PEG-DPPE), as described previously [17]. Briefly, 10 mg DPPE, 90 mg Mal-PEG-NHS and 3 μl triethylamine (Nacalai Tesque, Kyoto, Japan) were dissolved in 2 ml dichloromethane. The reaction mixture was stirred at room temperature (RT) for 24 h. The crude product was obtained by precipitation in diethyl ether (Nacalai Tesque, Kyoto, Japan). Mal-PEG-DPPE was extracted with chloroform (Nacalai Tesque, Kyoto, Japan) and dried. Mal-PEG-DPPE was obtained as a white powder (yield 76%).

Purification of sCR1

Human sCR1 was purified from the culture medium of CHO cells expressing sCR1 (CRL-10052TM, ATCC; Manassas, VA, USA), as previously described [19,20]. Briefly, CHO cells were cultured in α-MEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Equitech-Bio, Kerrville, TX, USA), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA), and 500 nM methotrexate (MTX) (Sigma-Aldrich, St. Louis, MO, USA). After the cells proliferated to confluence, they were washed three times with phosphate buffered saline (PBS) (Nissui Pharmaceutical (Tokyo, Japan) and cultured in serum-
free ASF104 medium (Ajinomoto Co. Inc., Tokyo, Japan) supplemented with 500 nM MTX for an additional 2 days. The culture medium was collected and centrifuged at 1000 rpm for 5 min, the supernatant filtered through a membrane filter (pore size: 0.45 \( \mu \)m) (Milipore™, MA 01821, USA). sCR1 was purified from the culture medium by affinity chromatography using a HiTrap Heparin HP column (GE Healthcare, Buckinghamshire, UK). The sCR1 concentration in the eluent was determined using a micro BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA).

The protective effect of sCR1 on islets from xenoreactive antibodies and complement was examined. Rat islets were incubated in 50% rabbit serum supplemented with various amounts of sCR1 (0-1000 \( \mu \)g/ml). Islet morphology was observed under a phase-contrast microscope (IX71, Olympus Optical Co. Ltd., Tokyo, Japan) with time. Sera were collected to determine insulin leakage from destroyed islets. Insulin concentrations in the sera were determined by ELISA (Shibayagi, Gunma, Japan).

**Immobilization of sCR1 on the islet surface using Mal-PEG-lipid**

Islets were isolated from ACI/N rats (male, 8 week old, Shimizu Co., Japan). A collagenase (Nitta Gelatin, Osaka, Japan) solution was injected into the pancreas through the pancreatic duct and left for 19 min at 37°C for digestion. Islets were isolated by centrifugation using a discontinuous density gradient of Ficoll/Conray solutions [21]. Islets were maintained in culture medium (RPMI-1640, Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, 100 U/ml penicillin, and 100 \( \mu \)g/ml streptomycin) for 7 days to remove damaged cells before use.

sCR1 was immobilized on the surface of islets using Mal-PEG-DPPE [22], as outlined in Scheme 1. Thiol groups were introduced onto sCR1 by thiolation using Traut’s reagent [18,20]. Briefly, 1 ml sCR1 solution (2 mg/ml) was mixed with 100 \( \mu \)l
Traut’s reagent (10 mg/ml) (MP Biomedicals, Illkirch, France). The solution was agitated at RT for 1 h. Thiolated sCR1 (sCR1-SH) was purified using a Sephadex G25 column (GE Healthcare, Buckinghamshire, UK). The number of thiol groups introduced was determined by Ellman’s reagent (Nacalai Tesque, Kyoto, Japan). Approximate 10 thiol groups were introduced into each sCR1 molecule under this condition. Islets (100 islets) were incubated in a solution of Mal-PEG-DPPE (1 ml, 1 mg/ml in PBS) for 20 min at RT and then washed 3 times with PBS. Islets modified with Mal-PEG-DPPE (Mal-PEG-islets) were obtained. Mal-PEG-islet was mixed with

Scheme 1. Immobilization of sCR1 on the pancreatic islet surface using Mal-PEG-DPPE. (A) Introduction of thiol groups to sCR1 by Traut’s reagent; (B) The molecular structure of Mal-PEG-DPPE; (C) Immobilization of sCR1 through maleimide/thiol reaction on the islet cell membrane.
1 ml of sCR1-SH solution (1 mg/ml in PBS) and left for 40 min at RT. After the islets were washed with PBS three times, sCR1-immobilized islets (sCR1-PEG-islets) were obtained.

Thiol groups were introduced to bovine serum albumin (BSA) by mixing 1 ml BSA solution (2 mg/ml in PBS) with 100 μl Traut’s reagent (10 mg/ml) for 1 h at RT. Thiolated BSA was purified by a PD-10 column. BSA-immobilized islets (BSA-PEG-islets) were prepared following the method for sCR1 immobilization mentioned above.

**Immunostaining of sCR1 on islets**

Immunostaining for surface sCR1 on islets was carried out using mouse anti-sCR1 antibody (J3D3, Invitrogen, Carlsbad, USA) and Alexa 488-conjugated goat anti-mouse antibody (Beckman Coulter, USA). sCR1-PEG-islets were incubated in 2 ml of an anti-sCR1 antibody solution (1 μg/ml in culture medium) for 1 h at 37°C and washed with culture medium. The islets were further incubated with 2 ml Alexa 488-conjugated anti-mouse antibody solution (1 μg/ml in culture medium) for 1 h at 37°C and washed 5 times with culture medium. The islets were then observed by confocal laser-scanning microscopy (FLUOVIEW FV500, Olympus, Tokyo, Japan).

**Surface plasmon resonance (SPR) monitoring of sCR1 binding to Mal-PEG-DPPE**

Reaction of sCR1-SH with a maleimide group of Mal-PEG-DPPE was monitored using a housemade surface plasmon resonance (SPR) apparatus [23]. BK7 glass plates were cleaned with piranha solution (70% concentrated sulfuric acid and 30% hydrogen peroxide) for 10 min at RT, washed with distilled water and dried under a stream of dried nitrogen gas. The cleaned glass plate was mounted on a rotating stage of a thermal evaporation coating apparatus (V-KS200, Osaka Vacuum,
CHAPTER 3 Immobilization of sCR1 on islets

Ltd., Osaka, Japan). A 1 nm chromium layer and 49 nm gold layer were sequentially coated onto the glass plates. The gold-coated glass plate was immersed in a 1 mM 1-dodecanethiol (Wako Pure Chemical, Osaka, Japan) solution in ethanol to form a self-assembly monolayer (CH$_3$-SAM) which facilitates the binding with Mal-PEG-DPPE via hydrophobic interaction. The glass plate was coupled to a hemicylindrical prism of SPR with immersion oil (n = 1.515, Cargille Laboratories, Cedar Grove, NJ).

SPR measurements were performed at 37°C. A $p$-polarized He-Ne laser light ($\lambda = 632.8$ nm) was irradiated to the substrate surface from the back side through the prism. The intensity of the reflected light was monitored while a solution of Mal-PEG-DPPE (100 $\mu$g/ml in PBS) flowed onto the CH$_3$-SAM surface for 30 min, washed with PBS for 20 min, and sCR1-SH solution (100 $\mu$g/ml in PBS) was then applied for 40 min. As a control experiment, sCR1-SH solution was applied to the CH$_3$-SAM surface with Mal-PEG-DPPE treated with 1 mM cysteine.

Protection of islets from xenoreactive antibodies and complement

Blood drawn from a NZW rabbit (male, 20 week-old, Shimizu Co., Japan) was allowed to clot for 30 min at RT in a glass test tube and then was centrifuged at 2000 rpm for 30 min at 4°C. Serum was collected as the supernatant, pooled and filtered through a membrane filter (200 nm pore size). Serum was stored at -80°C until use. The prepared rabbit serum was used for an antibody/complement-mediated cytotoxicity assay. Rabbit serum without complement activity was also prepared by heating the serum at 58°C for 30 min.

sCR1-PEG-islets, BSA-PEG-islets, and naive islets were incubated in 50% rabbit serum diluted with PBS to observe the protective effect of sCR1 immobilized on islets against xenoreactive antibodies and complement [24]. Islet morphology during serum incubation was observed under a phase-contrast microscope (IX71,
Olympus Optical Co. Ltd., Tokyo, Japan) with time. Sera were collected to observe insulin leakage from destroyed islets. Insulin concentrations in the sera were determined by ELISA. To see activation of the complement system, C3a concentration in the sera were determined by ELISA (USCN Life Science Inc., Wuhan, China).

Live and dead staining were carried out using a tetramethylrhodamine, ethyl ester, and perchlorate (TMRE) solution (Invitrogen, Carlsbad, CA, USA) and a Yoyo-1 solution (Invitrogen, Carlsbad, CA, USA). Four micro liters of the TMRE mixture and 0.5μl the Yoyo-1 solution were added to 20 islets in 1 ml culture medium. The islet suspension was incubated for 30 min at RT. After staining, the islets were washed 3 times in culture medium and then observed with confocal laser-scanning microscopy.

**Glucose-stimulated insulin secretion from islets**

A static glucose stimulation test was conducted to examine the insulin secretory function of modified islets [25]. Fifty sCR1-PEG-islets, BSA-PEG-islets, or naive islets, were sequentially incubated in Krebs-Ringer solution (KRB) with glucose concentrations of 0.1 g/dl, 0.3 g/dl, and 0.1 g/dl for 1 h each at 37°C. The insulin secreted into the KRB was determined by ELISA. Islets that were exposed to 50% rabbit serum for 24 h were also subjected to the static glucose stimulation test.

**Statistical analysis**

Comparisons between two groups were performed using Student’s t-test. \( P < 0.05 \) was considered significant. All statistical calculations were performed using JMP software version 5.1.1.
RESULTS

We immobilized sCR1-SH onto the islet surface through Mal-PEG-DPPE. Thiol groups were introduced on sCR1 using Traut’s reagent (Scheme 1A). Mal-PEG-DPPE was incorporated into the cell membrane of islets through hydrophobic interaction [14-19], and sCR1-SH was immobilized on the islets through the reaction between thiol groups of sCR1-SH and maleimide groups on islets (Scheme 1C).

Protective effect of sCR1

sCR1 was purified from the culture medium of CHO cells. Its protective effect against xenoreactive antibodies and complement was examined using rat islets and rabbit serum. Rat islets were incubated in 50% rabbit serum supplemented with different concentrations of sCR1 (0 – 1000 μg/ml). After 24 h incubation, morphologies of islets observed under a phase contrast microscope and the amounts of insulin leaked from damaged β-cells are summarized in Fig. 1A and Fig. 1B, respectively. Many swelled and damaged cells were found at the periphery of rat islets that were incubated in 50% rabbit serum without sCR1 for 24 h. A large amount of insulin (40.5 ng/islet/24 h) was detected in serum. Because rat and rabbit are a discordant animal combination [26], preformed xenoreactive antibodies against rat exist in rabbit serum. Fig. 1 includes photos of islets incubated in 50% heat inactivated serum, which loses complement activity. No damaged cells were found on the islet. The results indicate that the antibodies form immune complexes on rat islets and the complex activates the complement system, resulting in formation of membrane attack complexes on cells within the islets. When islets were incubated in
50% rabbit serum supplemented with different amounts of sCR1, numbers of swelled cells on the islets and amounts of insulin leaked from damaged β-cells decreased in a dose-dependent manner with respect to sCR1. sCR1 purified from CHO culture medium can control the activation of the complement system.

**Figure 1.** The protective effect of islets by sCR1 against xenoreactive antibodies and complement. Rat islets were incubated in 50% rabbit serum supplemented with various amounts of sCR1. (A) Representative islet morphology: (A-1) Naïve islet; (A-2) Islet incubated in 50% heat-inactivated rabbit serum for 24 h; (A-3) Islet incubated in 50% rabbit serum; (A-4) Islet in 50% rabbit serum supplemented with sCR1 (250 µg/ml). (B) Amounts of insulin leaked from islets in 50% rabbit serum supplemented with various amounts of sCR1. * indicates statistical significance ($P < 0.05$).

**SPR monitoring of sCR1 binding to Mal-PEG-DPPE**

The reaction between sCR1-SH and a maleimide group of Mal-PEG-DPPE was monitored by the SPR method. An SPR sensor carrying CH$_3$-SAM and sCR1-SH with 10 thiol groups/molecule were used. When a Mal-PEG-DPPE solution was applied to the CH$_3$-SAM surface, SPR angle increased with time (Fig. 2A), indicating Mal-PEG-DPPE was spontaneously immobilized on the CH$_3$-SAM surface. As a solution of sCR1-SH was applied to the Mal-PEG-DPPE immobilized sensor surface,
the SPR angle again increased with time (Fig. 2B), indicating sCR1 binding on the sensor surface. However, when the sensor surface was pre-treated with 1 mM cysteine solution, only a small increase in the SPR angle was observed. Maleimide groups on the sensor surface were consumed by cysteine. These results indicate that sCR1-SH can be immobilized on an islet surface covered by Mal-PEG-DPPE through the thiol-maleimide reaction.

**Figure 2.** Real-time monitoring of the reaction between sCR1-SH and maleimide groups of Mal-PEG-DPPE immobilized on the SPR sensor surface. (A) A Mal-PEG-DPPE solution was applied to an SPR sensor surface covered with CH3-SAM; (B) Line A: a sCR1-SH solution was infused onto the Mal-PEG-DPPE–immobilized surface. Line B: SPR angle increase on the Mal-PEG-DPPE–immobilized surface, which was pretreated with 1 mM cysteine.

**Immobilization of sCR1 on the islet surface**

Islets were sequentially immersed in Mal-PEG-DPPE and sCR1-SH solutions. The presence of sCR1 on the islet surface was examined by immunohistochemical staining using anti-sCR1 antibody. As shown in Fig. 3(A-4), bright green fluorescence
was observed at the periphery of islets treated with Mal-PEG-DPPE and sCR1-SH, but not naïve islets. These islets were also examined under a phase contrast microscope. No morphological differences were observed before or after sCR1 immobilization.

**Figure 3.** Immobilization of sCR1 on islets using Mal-PEG-DPPE. (A) Phase contrast microscopic image and immunohistochemical staining of sCR1. (A-1) and (A-2): Naïve islets; (A-3) and (A-4): sCR1-islets; (B) Glucose stimulation test. Black: Naïve islets; Grey: BSA-islets; White: sCR1-islets.

Static glucose stimulation tests were carried out to examine the effect of sCR1 immobilization on the ability of islets to secrete insulin (Fig. 3B). Naïve islets, BSA-islets, and sCR1-islets could control the amounts of insulin secreted in response to changes in glucose concentration. Although slightly less insulin was secreted by modified islets, BSA-islets, and sCR1-islets, than naïve islets under 0.3 g/dl glucose, the difference was not statistically significant.
Protective effect of sCR1 immobilized on islet surface

The protective effect of immobilized sCR1 on islets against xenoreactive antibodies and complement was examined. After 24 h incubation of naïve, BSA-, and sCR1-islets in 50% rabbit serum, their morphologies were examined under a phase contrast microscope (Fig. 4). The numbers of swelled and damaged cells on naïve islets were counted and compared.

**Figure 4.** The protective effect of immobilized sCR1 on islets against xenoreactive antibodies and complement. (A): Morphological changes of BSA-islets and sCR1-islets incubated in 50% rabbit serum. (B): Immunohistochemical staining for sCR1 after incubation of sCR1-islets in 50% rabbit serum for 24 h. Scale bar, 200 μm. (C): Live (TMRE (red)) and dead (Yoyo-1 (green)) staining of islet cells. Cell viability was examined (C1) before and (C2) after incubation of islets in 50% rabbit serum for 24 h. The islets were observed using confocal laser-scanning microscopy. Scale bar, 50 μm.
islets and BSA-islets increased with time. However, such swelled and damaged cells were rarely found on sCR1-islets. Fig. 4 also includes immunohistochemical staining of sCR1 after incubation of sCR1-islets in 50% rabbit serum for 24 h. After 24 h incubation, sCR1 still existed on the islets’ surface and protected islets from xenoreactive antibodies and complement.

Islets were treated with TMRE (red) and Yoyo-1 (green) to examine islet viability [31]. As shown in Figure 4(C1), sCR1-PEG-islets were clearly stained, most of cells were stained red with TMRE, but few cells stained green with Yoyo-1, indicating a lack of damaged cells in naïve islets, BSA or sCR1 immobilized islets. After 24 h incubation in 50% rabbit serum of naïve islets, sCR1-PEG-islets, and BSA-PEG-islets, live and dead staining was also carried out. Figure 4(C2) shows that strong green fluorescence was observed on naïve islets and BSA-PEG-islets, indicating that islet cells were severely damaged during incubation in rabbit serum. In contrast, islets incubated in 50% heat-treated rabbit serum displayed patterns similar to islets before incubation. It suggested that the complement activation was responsible cell damage. A few green cells were found at the periphery of sCR1-PEG-islets which were incubated in 50% rabbit serum for 24 hrs, while most islets were stained red. sCR1 immobilization on islet surface exerted effective protection on islets by the suppression of complement activation in rabbit serum.

Insulin concentrations in media were also measured to determined extent of damage to β-cells by determining the amount of insulin leakage from dead β-cells. The amounts of leaked insulin are plotted with islet incubation time in 50% rabbit serum (Fig. 5B). After 24 h, significantly larger amounts of insulin were found in the conditioned serum of naïve islets and BSA-islets than that of sCR1-islets. Fig. 5B also includes the amounts of insulin leaked from naïve islets incubated in heat-inactivated
serum. These results indicate that sCR1 on islets effectively protects islets from xenoreactive antibodies and complement.

![Graph](image-url)

**Figure 5.** Damage of islets through the complement activation. (A): Amounts of insulin leaked from naïve islets (closed circles), BSA-islets (closed squares), and sCR1-islets (open squares) during incubation in 50% rabbit serum, as well as from naïve islets incubated in 50% heat-inactivated serum (open circles). * indicated statistical significance compared with naïve islets. (*P* < 0.05). (B) Amounts of C3a generated during incubation of different kinds of islets in 50% rabbit serum.

We then examine the contribution of complement to the destruction of naïve rat islets in rabbit serum and protective effect of immobilization of sCR1 on islets from the attack by the complement system. Concentrations of C3a which is generated when the complement system is activated were determined. As shown in Fig. 5B, a marked increase in C3a (6.22 ng/ml) was observed in serum after incubation with naïve islets as compared to serum without islet incubation (0.53 ng/ml). When sCR1-PEG-islets and BSA-PEG-islets were incubated in 50% rabbit serum, the concentrations of C3a generated were 0.64 ng/ml and 7.01 ng/ml, respectively. These results indicated that the immobilized sCR1 could completely inhibit complement...
activation on the islet surface and effectively protect the islets from complement cytotoxicity.

After islets were incubated 24 h in 50% rabbit serum, the ability of naïve, BSA-, and sCR1-islets to release insulin was examined using the glucose stimulation test (Fig. 6). Naïve islets could release a small amount of insulin, but could not increase insulin secretion in response to high glucose stimulation after 24 h incubation. Naïve islets incubated in heat-inactivated rabbit serum could regulate insulin release in response to changes in glucose concentration. These results indicate that xenoreactive antibodies in rabbit serum and complement exert cytotoxic effects on islets. However, sCR1-islets were able to release low level insulin at 0.1 g/dl glucose and increase their secretion level approximately 4.02 times to 0.3 g/dl, even after 24 h incubation in rabbit serum. BSA exerted a limited protective effect against xenoreactive antibodies and complement.

**Figure 6.** Glucose stimulation tests after incubation of islets in 50% rabbit serum for 24 h. ■: Naïve islets incubated in 50% heat-inactivated rabbit serum, □: naïve islets, ■: BSA-islets, and □: sCR1-islets incubated in 50% rabbit serum for 24 h, * indicates statistical significance ($P < 0.05$).
DISCUSSION

In clinical islet transplantation, IBMIR is a central problem [5,6]. Exposure of islets to fresh blood activates the coagulation cascade and innate immune responses, resulting in islet destruction by nonspecific inflammatory reactions. Surface modification of islets with water soluble polymers and immobilization of antithrombogenic enzyme, thrombomodulin, and urokinase, on islet surface enables modified islets to inhibit IBMIR [14-18,27,28]. The complement system is a part of innate immunity and is activated in a cascade manner when exotic materials or non-self tissues are introduced [29]. Once complement is activated, surface-bound C3b activates the amplification loop to generate a vast number of C3b molecules and trigger the assembly of the membrane attack complex (MAC), inducing the lysis of target cells. This activation also leads to the generation of anaphylatoxins (C3a and C5a), which in turn activate and recruit inflammatory cells such as phagocytes and lymphocytes. Several studies have shown that the inhibition of complement activation not only downregulates the inflammatory response but also remarkably reduces platelet prothrombinase activity, which is essential to the coagulation process [13,30]. Complement activation is intimately linked to IBMIR. In addition, porcine islets have been examined because of the shortage of human pancreatic islet donors [7]. However, most human recipients have xenoreactive antibodies against porcine tissue. Xenoreactive antibodies and complement play a major role in hyperacute rejection in the context of xeno-transplantation [8]. It is crucially important to control complement activation in both alloislet and xenoislet transplantation.
sCR1 is able to inhibit complement activation in both classical and alternative pathways [11-13]. In this study, sCR1 was immobilized on the islet surface through Mal-PEG-DPPE. Our mild modification technique did not deteriorate cell viability or ability to secrete insulin. sCR1 on the islet surface effectively inhibits complement activation and protects islets against attack from xenoreactive antibodies and complement. Immobilization of sCR1 downregulates the inflammatory response and reduces platelet prothrombinase activity. In our previous study [28], immobilization of urokinase could reduce the number of islets necessary to normalize blood glucose levels from 250 to 125 in a mouse transplantation model. The co-immobilization of sCR1 and urokinase is likely to produce a synergistic effect; this possibility will be carefully examined in vivo.

It has been reported that a high-dose systemic infusion of sCR1 (approximately 40 mg/kg) ameliorates hyperacute xenograft rejection [13]. As mentioned above, sCR1 on the islet surface effectively inhibits complement activation and protects islets against attack by xenoreactive antibodies and complement. There are reasons that the immobilization of sCR1 on islets is superior to systemic infusion. Immobilization of sCR1 on the islet surface inhibits complement activation at the site where it should be controlled. Side effects that are expected to occur as a result of systemic infusion of sCR1 can be avoided in the context of sCR1 islet immobilization. However, the stability of sCR1 on islets is a major obstacle that remains to be overcome. sCR1 should persist on islets and control complement activation for as long as the islets exist, but PEG-DPPE and its conjugating enzyme are slowly released from the islet surface [18]. Increasing the stability of sCR1 on islets would significantly improve their utility, and should be pursued.
REFERENCES


CHAPTER 4

Layer-by-layer co-immobilization of soluble complement receptor 1 and heparin on islets

INTRODUCTION

Early graft loss is the major obstacle during transplantation of clinical islets of Langerhans (islets) [1]. When islets are transplanted through the portal vein to liver tissue in a clinical setting, exposure of the islets to blood triggers a thrombotic/inflammatory reaction [2]. Instant blood-mediated inflammatory reactions (IBMIRs) have been identified as the main reason for islet loss in the early post-transplantation stage [3]. Two to three donors per recipient are generally necessary to achieve insulin dependence [4]. Several experimental approaches have been proposed to prevent IBMIRs, including systemic treatment [5-8], and although systemic administration of drugs or proteins is effective, it is usually associated with a risk of complications and side effects. Therefore, local inhibition of the coagulation cascade has also been attempted [9-11].

We have also made various attempts [12-16] to suppress IBMIRs using amphiphilic polymers such as poly(ethylene glycol)–phospholipid conjugates (PEG-lipids) and poly(vinyl alcohol) carrying long alkyl side chains. These molecules
spontaneously incorporate into the cell membrane via hydrophobic interactions between the alkyl chains and the lipid bilayer of the cell membrane. In addition, various bioactive substances such as urokinase, thrombomodulin, and human soluble form complement receptor 1 (sCR1) have been immobilized on islets through the bilayer [17-20]; sCR1 is a potent inhibitor of both the classical and alternative complement activation pathways [21-23], and urokinase and thrombomodulin can inhibit formation of blood clots on the islets. In this study, we attempted to engineer both anti-thrombogenic and anti-complement properties in the islet cells by co-immobilizing sCR1 and heparin layer-by-layer, and the effects of these layers were examined *in vitro*. 
Purification and thiolation of sCR1

Human sCR1 was prepared using Chinese hamster ovary (CHO) cells expressing sCR1 (CRL-10052™, ATCC; Manassas, VA, USA) as described previously [24]. Briefly, CHO cells were maintained in α-MEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Equitech-Bio, Kerrville, TX, USA), 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen), and 500 nM methotrexate (Sigma-Aldrich, St. Louis, MO, USA) to form a confluent cell monolayer. Medium was then changed to ASF104 (Ajinomoto Co. Inc., Tokyo, Japan) supplemented with 500 nM methotrexate and cultured for an additional 2 days. sCR1 was purified from the supernatants by affinity chromatography using a HiTrap Heparin HP column (GE Healthcare, Buckinghamshire, UK), and the eluent was further purified with a centrifugal filter device (Amicon Ultra -15, 100 kDa; Milipore Corporation, MA, USA). The sCR1 concentration was determined using a micro BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA).

For thiolation, 1 mL of 3 mg/mL sCR1 solution was mixed with 100 μL of 10 mg/mL Traut’s reagent in phosphate-buffered saline (PBS; MP Biomedicals, Illkirch, France). The solution was left at room temperature for 1 hour, after which thiolated sCR1 (sCR1-SH) was purified by a Sephadex G25 column (GE Healthcare). Ellman’s reagent (Nacalai Tesque, Kyoto, Japan) was used to determine the number of thiol groups per sCR1 molecule.
Surface plasmon resonance (SPR) monitoring of sCR1-heparin interactions

The interaction between sCR1 and heparin was monitored by an in house-designed SPR instrument [25], with the SPR sensor surface consisting of a 1-nm chromium layer and a 49-nm gold layer on a BK-7 glass plate (refractive index 1.515; Arteglass Associates Co., Kyoto, Japan). A hydrophobic self-assembled monolayer (SAM) of 1-dodecanethiol (Wako Pure Chemical, Osaka, Japan) (CH₃-SAM) was formed on the gold-coated glass by incubation in 10 mM 1-dodecanethiol in ethanol at room temperature overnight. The glass plate was set on a prism of the SPR apparatus and the flow cell was assembled on the glass plate. The intensity of the reflected light was monitored in real-time when each solution was applied to the flow cell. Maleimide-poly(ethylene glycol)-conjugated phospholipid (Mal-PEG-DPPE) was synthesized as described previously [20]. After Mal-PEG-DPPE solution (100 µg/mL in PBS) was applied to the CH₃-SAM surface, sCR1-SH (100 µg/mL in PBS) was applied for 30 min at 37 °C. After washing with PBS, heparin (1 mg/mL in PBS) and sCR1 solutions (100 µg/mL in PBS) were alternately flowed for 30 min at 37 °C for each step.

Immobilization of sCR1 and heparin on the islet surface

Pancreases from ACI/N rats (8-week-old males; Shimizu Co., Japan) were digested by the collagenase method and islets were purified from the digested tissue using a discontinuous density gradient of Ficoll/Conray solutions [26]. Islets were maintained in culture medium (RPMI-1640; Invitrogen) supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin for 5 days.
Scheme 1. Immobilization of sCR1 and heparin on the islet cell surface.

sCR1 and heparin were immobilized on the islet surface as illustrated in Scheme 1. Islets were incubated in Mal-PEG-DPPE solution (1 mg/mL in PBS) for 20 min at room temperature to prepare the Mal-PEG-islets [20]. After washing with PBS, Mal-PEG-islets were incubated in a solution of sCR1-SH (1 mg/mL in PBS) for 1 hour at room temperature to allow sCR1 immobilization on the islet surface (sCR1-islets). sCR1-islets were washed three times with serum-free medium M199 and incubated in a heparin solution (5 mg/mL in medium M199) for 30 min at room temperature to prepare the first double-layer (sCR1-heparin) on the islet surface. Subsequent sCR1-heparin layers were deposited onto the first layer by incubating sCR1-heparin-islets in sCR1 (500 μg/mL in M199) and heparin (5 mg/mL in M199) alternately for 30 min at room temperature until the desired number of layers was achieved. Finally, the islets were washed with culture medium.
CHAPTER 4  Layer-by-layer co-immobilization of sCR1 and heparin

**Observation of sCR1 and heparin immobilized on islet surfaces**

Immunostaining of immobilized sCR1 was carried out using anti-sCR1 J3D3 antibodies (Invitrogen) as primary antibody and fluorescent-Alexa488 labeled anti-mouse antibody (Beckman Coulter, USA) as counterstain. Fluorescein isothiocyanate (FITC)-conjugated sCR1 (FITC-sCR1) was prepared for islet observation. Briefly, 1 mL of sCR1 solution (3 mg/mL) was mixed with 100 μL FITC (0.12 mg in 50 μL dimethyl sulfoxide; Dojindo Laboratories, Kumamoto, Japan) at a molar ratio of 1:20. The mixture was agitated at room temperature for 6 hours. FITC-sCR1 was separated from un-reacted reagent using a Sephadex G25 column and stored in the dark at 4 °C until use. Stained islets were observed with confocal laser scanning microscopy (FLUOVIEW FV500, Olympus, Tokyo, Japan).

FITC-heparin was prepared as described previously [13]. One milliliter of heparin (40 mg/mL in pure water) was mixed with FITC (2.6 mg in 100 μL dimethyl sulfoxide) and the mixture was left at room temperature overnight. The crude product was precipitated in an 8:2 mixture of acetone and diethylether, evaporated, and washed three times with acetone. The precipitate was then dialyzed against PBS for 2 days using dialysis cassettes (3500 Da; Thermo Scientific) to obtain FITC-heparin.

**Anti-thrombin activity of immobilized sCR1-heparin**

To evaluate the anti-thrombin activity of sCR1-heparin-immobilized substrate surfaces, a silicon sheet (10 mm thick) with a hole (6 mm diameter, 4 wells) was placed on a CH₃-SAM glass plate. One hundred microliters of Mal-PEG-DPPE solution (500 μg/mL in PBS) was applied to each well and left for 30 min at room temperature. The
wells were washed five times with PBS. One hundred microliters of sCR1-SH (100 μg/mL in PBS) was applied to each well and left for 1 hour at room temperature. After washing with PBS, heparin (1 mg/mL in PBS) and sCR1 solution (100 μg/mL in PBS) were sequentially applied and left for 30 min after each step until the desired number of sCR1-heparin layers was formed. As a control, a solution of cystein (1 mM in PBS) was added to the Mal-PEG-DPPE-immobilized wells and left for 1 hour.

Anti-thrombin activity was examined via enzyme-linked immunosorbent assay (ELISA) using the Sensolyte® 520 thrombin activity assay kit (AnaSpec, CA, USA) with a slight modification. Tris-HCl buffer (100 μL) with or without antithrombin III (10 μg/mL) and 50 μL thrombin solution (1 μg/mL in assay buffer) was sequentially added to each well and the plate was incubated for 10 min at 37 °C. Fifty microliters of the thrombin-substrate solution were then added to each well. The reaction mixtures were incubated for 30 min at 37 °C and the fluorescence (excitation 490 nm, emission 520 nm) was measured by a fluorophotometer (F-2500; Hitachi, Co., Tokyo, Japan).

**Protective effect of sCR1 from complement-mediated cytotoxicity**

The protective effect of immobilized sCR1 from antibody/complement-mediated cytotoxicity was examined as described previously [19, 20]. Blood was drawn from a New Zealand white rabbit (20-week-old male; Shimizu Co.), applied to glass test tubes, and allowed to clot for 30 min at room temperature. The tubes were centrifuged at 2000 rpm at 4 °C for 30 min. Serum was collected as the supernatant, pooled, filtered through a 200-nm membrane filter, and stored at -80 °C until use. The complement in the rabbit serum was inactivated by heating the serum at 56 °C for 30 min. Twenty islets from each
group were incubated in 1 mL of 50% rabbit serum at 37 °C. Serum was exchanged every 24 hours. Islet morphology was observed every 24 hours under a phase contrast microscope (IX71, Olympus Optical Co. Ltd., Tokyo, Japan). Insulin levels in the collected sera were determined by ELISA (Shibayagi, Gunma, Japan).

**Insulin secretion by modified islets**

A static glucose stimulation assay was carried out to determine whether insulin secretion function deteriorates following immobilization of sCR1 and heparin [27]. Fifty islets were sequentially incubated in Krebs-Ringer buffer containing 0.1 g/dL or 0.3 g/dL glucose, with each incubation proceeding for 1 hour at 37 °C. Supernatants were collected and insulin concentrations were determined by ELISA.
CHAPTER 4  Layer-by-layer co-immobilization of sCR1 and heparin

RESULTS

SPR monitoring of sCR1-heparin interactions

Interactions between sCR1 and heparin on a gold-coated substrate were monitored by an SPR apparatus (Fig. 1A). When Mal-PEG-DPPE was applied to the CH$_3$-SAM surface, an increase in SPR signal was observed (Fig. 1A), reflecting the immobilization of Mal-PEG-DPPE through the hydrophobic interaction between the alkyl chains of DPPE and CH$_3$-SAM [20]. When sCR1-SH with an average of 10.8 thiol groups per

![Image](image1.png)

Figure 1. sCR1 and heparin layer formation via affinity-based interactions. (A) Monitoring of sCR1 and heparin layer formation by SPR. A Mal-PEG-DPPE solution was applied to CH$_3$-SAM on a gold-coated glass plate, sCR1-SH was applied, and heparin and sCR1 were sequentially applied to the surface. (B) The amounts of sCR1 in the layers was determined by BCA assay. (sCR1 and heparin) is one double-layer (DL).
molecule was applied, (Fig. 1A, first arrow), the SPR signal increased, demonstrating binding of sCR1 to the maleimide group of Mal-PEG-DPPE. The SPR signal underwent step-by-step increases following each application of heparin or sCR1 solutions (arrows, Fig. 1A).

The amounts of sCR1 immobilized after the formation of each double-layer of sCR1 and heparin were determined by the BCA assay (Fig. 1B). The amounts of sCR1 increased with an increase in the number of double-layers (Fig. 1B), an observation consistent with the SPR shifts in Fig. 1A. This result demonstrates the formation of multiple layers of sCR1 and heparin on the CH3-SAM surface.

**Immobilization of sCR1 and heparin on islet surfaces**

Immobilized sCR1 and heparin layers were introduced layer-by-layer to islet cell surfaces (Scheme 1). Cells were treated with Mal-PEG-DPPE to introduce maleimide groups on the surface [20], and sCR1-SH was immobilized on the Mal-PEG-treated islet surface by the reaction between thiol and maleimide. Heparin and sCR1 were alternately added to the surface to form multiple layers. Immunostaining of sCR1 revealed the presence of sCR1 on the islets, as indicated by fluorescence at the islet peripheries (Fig. 2, panel A1). No fluorescence was observed on islets treated with sCR1 without thiolation (Fig. 2, panel A2). As shown in Fig. 2 (panels B1 and B2), FITC-heparin fluorescence was observed on the surface of sCR1-islets (Fig. 2, panel B1) but no clear fluorescence was detected on naïve islet surfaces (Fig. 2, panel B2), indicating that heparin was immobilized via the interaction with sCR1 molecules on the islets.
Figure 2. Immobilization of sCR1 and heparin on islet surfaces. (A) Immunostaining of sCR1 on sCR1-islets (A1) and naive islets (A2). (B) sCR1-islets (B1) and PEG-islets (B2) were exposed to FITC-heparin. All islets were observed via confocal laser scanning microscopy. (C) Phase contrast image of islets immobilized with three sCR1-heparin double-layers after 3 days of culture. Scale bar: 200 μm.

We used FITC-sCR1 to examine the stability of multiple sCR1-heparin layers on the islets (Fig. 3). The fluorescence intensity increased as the number of layers increased, indicating that more sCR1 could be immobilized by increasing the number of layers on the islets. Immobilized sCR1 gradually disappeared from islet surface in all cases over time (Fig. 3). As expected, the retention time of sCR1 increased when the number of layers increased; islets with three sCR1-heparin layers maintained intact morphology after three days of culture, indicating that the layer-by-layer method did not influence islet viability.
Figure 3. Retention of sCR1 on islet surfaces. Multiple sCR1-heparin layers were formed by sequential exposure of sCR1-islets to heparin and sCR1 solutions (1L = one layer, 2L = two layers, and so on). FITC-sCR1 was used to visualize immobilized sCR1. Islets with multiple layers of sCR1 and heparin were observed by confocal laser microscopy after the islets were maintained in culture medium for the indicated periods. Scale bar: 200 μm.

<table>
<thead>
<tr>
<th>sCR1-1L</th>
<th>sCR1-2L</th>
<th>sCR1-3L</th>
<th>sCR1-4L</th>
<th>sCR1-5L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>Day 1</td>
<td>Day 2</td>
<td>Day 3</td>
<td></td>
</tr>
</tbody>
</table>

**Anti-thrombin activity of immobilized heparin**

The anti-thrombin activity of heparin immobilized on glass plates (heparin composed the outermost layer) by the layer-by-layer method was determined (Fig. 4). Anti-thrombin activity was detected neither on PEG-treated substrate nor on the sCR1-PEG surface. For the substrate surfaces treated with one or three sCR1-heparin layers,
however, the anti-thrombin activity was significantly increased. The immobilized heparin was able to interact with anti-thrombin and activate it to inhibit thrombin. No substantial differences in heparin activity were observed between surfaces with one or three sCR1-heparin layers just after preparation (Fig. 4). The activity of heparin in one double-layer gradually decreased with time (Fig. 4), reflecting the detachment of heparin from the surface. However, no substantial decreases in activity were observed for three-layer constructs (Fig. 4), suggesting that the layer-by-layer method improves heparin stability and provides an anti-thrombin effect for at least eight days.

![Figure 4](image)

**Figure 4.** Relative thrombin inactivation activities of multiple sCR1-heparin layers on glass plates. The activities were determined after the glass plates were maintained in culture medium (medium RPMI-1640 containing 10% FBS) for the indicated periods.

**Protective effect of immobilized sCR1 from complement-mediated cytotoxicity**

We exploited the fact that rabbit serum contains preformed antibodies against rat antigens to examine complement-mediated cytotoxicity in our system. Islets, sCR1-islets, sCR1-heparin-islets, and islets with three sCR1-heparin layers ((sCR1-heparin)$^3$-islets) were
incubated in 50% rabbit serum; the outermost layer of sCR1-heparin-islets and (sCR1/heparin)$^3$-islets consisted of heparin. The morphologies of these islets were observed over time via phase contrast microscopy (Fig. 5). Naïve islets were rapidly destroyed; a number of swelled cells were already detectable after 1 hour of incubation in 50% rabbit serum, and more than half of the cells had swelled after 24 hours of incubation (Fig. 5). Most islets were completely destroyed within 48 hours (Fig. 5). When we incubated islets in 50% heat-inactivated rabbit serum, inactivating the

![Figure 5](image-url)

**Figure 5.** Protective effects of sCR1 from antibody/complement cytotoxicity. (A) Phase contrast microscopy of sCR1-islets, sCR1-heparin-islets, (sCR1-heparin)$^3$-islets, and naïve islets maintained in 50% rabbit serum for the indicated periods. Images of islet morphology were taken before (a) or after (b) incubation in 50% heat-inactivated rabbit serum. Scale bar: 200 μm.
complement system, no islet damage was observed after 24 hours of incubation (Fig. 5, panels (a) and (b). sCR1-islets, sCR1-heparin-islets, and (sCR1-heparin)\(^3\)-islets maintained their morphologies for a longer time than naïve islets (Fig. 5). After 48 hrs, a small number of damaged cells were found at the periphery of the islets.

**Figure 6.** Amounts of insulin leakage from the islets into 50% rabbit serum every 24 hours.

When naïve islets were incubated in 50% rabbit serum for 24 hours, a large amount of insulin (498.8 ng/20 islets) was found in the supernatant (Fig. 6). During the second 24-hour period, however, the amount of leaked insulin decreased, since only a small number of viable islets remained (Fig. 6). When islets were incubated in 50% heat-inactivated rabbit serum, we detected 6.45 ng of insulin from 20 islets, an amount comparable to the physiological secretion of insulin by islets. During the first 24-hour incubation in 50% rabbit serum, the amounts of insulin leaked from sCR1-islets, sCR1-heparin-islets, and (sCR1-heparin)\(^3\)-islets were 4.3 ng, 7.2 ng, and 56.8 ng per 20 islets, respectively, with the sCR1-islet and sCR1-heparin-islet values at the same level as that of naïve islets incubated in heat-inactivated serum. Thus, the islets were effectively
protected from antibody/complement-mediated cytotoxicity by the immobilized sCR1. A relatively high insulin leakage was observed in (sCR1-heparin)$^3$-islets due to the slight damage of these cells. Substantial insulin leakages were observed in sCR1-islets, sCR1-heparin-islets, and (sCR1-heparin)$^3$-islets during a third 24-hour incubation (Fig. 6). However, insulin leakage decreased with increasing durations of sCR1 immobilization on the islets; the protective effect of sCR1 was observed more clearly with (sCR1-heparin)$^3$-islets than with sCR1-heparin-islets (Fig. 6).

**Glucose-stimulated insulin secretion**

Finally, we examined the effects of sCR1 and heparin immobilization on insulin release (Fig. 7). As the glucose concentration in Krebs-Ringer buffer increased from 0.1 g/dL to 0.3 g/dL, the insulin secretion of naïve islets increased 17.7-fold. Similar insulin

![Graph showing glucose-stimulated insulin secretion](image)

**Figure 7.** Static glucose stimulation of naïve islets, sCR1-islets, and islets immobilized with one, three, or five double-layers of sCR1-heparin. Insulin concentrations were determined by ELISA.
release behaviors were observed one-layer through five-layer sCR1-heparin-islets (Fig. 7). No substantial differences were observed between any groups of islets.
DISCUSSION

In the present study, we modified sCR1 to carry thiol groups and immobilized it to islets via maleimide-PEG-lipid (Scheme 1). Heparin was co-immobilized on the islets with sCR1 by simple addition of a heparin solution to the islet suspension, because sCR1 has a strong affinity for heparin [24, 28, 29]. After formation of the first double-layer of sCR1-heparin, additional double-layers were easily formed layer-by-layer by simply repeatedly alternating the solutions. Previously, heparin was immobilized onto islet surfaces via avidin, leading to the evasion of IBMIR [10]. Although effective, this method is hardly applicable to human patients because avidin is a xenogeneic protein isolated from chicken eggs [30] and thus may cause unfavorable immune reactions.

We aimed to inhibit blood coagulation and complement activation to prevent early islet graft loss caused by IBMIR. Heparin and sCR1, a potent inhibitor of the classical and alternative complement activation pathways, were alternately immobilized on islets (Scheme 1). Although heparin is known to lose anti-thrombin activity when complexed with molecules such as protamine [31], our system demonstrated anti-thrombin activity (Fig. 4) and protective effects from antibody/complement cytotoxicity (Fig. 5 and Fig. 6) regardless of the number of sCR1-heparin layers formed on the islets.

There is no consensus on the period required to inhibit blood coagulation and complement activation to prevent early islet graft loss. We aimed to increase the retention period of heparin and sCR1 on islets with increasing numbers of sCR1-heparin double-layers. Heparin in three double-layers exhibited stronger anti-thrombin activity than that
in one double-layer after 8 days of culture (Fig. 4). sCR1 immunostaining, however, did not reveal a clear relationship between sCR1 retention period and the number of double-layers (Fig. 3). sCR1, however, was clearly seen on the islets regardless of the number of double-layers, and the protective effect of immobilized sCR1 was detectable after two days of culture.

The first two days following transplantation are the most severe for islets due to IBMIR, specifically the activation of the coagulation system and the complement cascade [2, 3]. A large quantity of islets was destroyed in this period. Although immobilized sCR1 and heparin were gradually released from the cell surface over several days, our approach will provide beneficial effects for clinical islet transplantation.
REFERENCES


CHAPTER 5

Microencapsulation of islets with living cells using polyDNA-PEG-lipid conjugate

INTRODUCTION

The bioartificial pancreas, which encapsulates islets of Langerhans (islets) within a semi-permeable membrane, is one of the therapeutic devices for patients with insulin-dependent diabetes mellitus (type I diabetes). It is a safe and simple way to transplant islets without the need for immuno-suppressive therapy. The semi-permeable membrane protects the islets from the immune system of a recipient patient, and thus the islets are expected to survive and release insulin for a long period of time and thereby control glucose metabolism. Various types of bioartificial pancreas have been proposed and developed [1-3], with the microencapsulated type being a promising model. In this example, islets are microencapsulated within an alginate/poly(L-lysine) polyion complex membrane [4] or an agarose hydrogel [5-8]. Our group has used the agarose system and has demonstrated its efficacy in diabetic animals [5-8]. Recently, we also have developed an original design for a bioartificial pancreas for transplantation into the liver through the portal vein [9-15]. However, materials comprising a bioartificial pancreas have not been
ideal and might activate defense reactions against foreign materials. Compatibility of the membrane with the recipient patient should be improved.

We proposed to enclose islets from a donor under a layer of cells from a recipient to increase compatibility with the patient. Since the outermost surface is the recipient’s own cells, the host immune defense system will not be provoked. In our previous study, we developed a method to enclose islets with living cells [15]. Amphiphilic poly(ethylene glycol)-conjugated phospholipid derivatives (PEG-lipid) and biotin/streptavidin reactions were employed. Although the biotin/streptavidin reaction worked well to cover the islets with living cells, streptavidin is a xenogeneic protein and is expected to activate the host immune system. We sought to improve this technique with the use of biocompatible materials.

In this study, we employed DNA hybridization instead of the biotin/streptavidin reaction. Polyadenine (polyA) and polythymine (polyT) were introduced onto the surfaces of the islets and HEK 293 cells, respectively, by using polyA or polyT-conjugated PEG-lipid. We already succeeded in the cell-cell attachment induced by hybridization of DNA-conjugated PEG-lipid [16]. The hybridization of DNA-conjugated PEG-lipid was effectively used for the cell-cell attachment and cell-immobilization to the substrate. The HEK 293 cells were thereby immobilized on the surface of the islets through hybridization of polyA and polyT. A layer of living HEK cells was formed on the surface of islets.
EXPERIMENTAL

Materials

$\alpha$-N-Hydroxysuccinimidyl-$\omega$-maleimidyl poly(ethylene glycol) (NHS-PEG-Mal, Mw: 5000) was purchased from Nektar Therapeutics (San Carlos, USA). 1, 2-Dipalmitoyl-sn-glycerol-3-phosphatidylethanolamine (DPPE) was purchased from NOF Corporation (Tokyo, Japan). Dichloromethane, triethylamine, sucrose and diethyl ether were purchased from Nacalai Tesque (Kyoto, Japan). Hoechst 33342 nuclear stain was purchased from Dojindo Laboratories (Kumamoto, Japan). Alexa 488-labeled goat anti-guinea pig IgG, minimum essential medium (MEM), HEPES buffer solution, Hanks’ balanced salt solution (HBSS), Medium 199, RPMI-1640 medium, penicillin, and streptomycin were purchased from Invitrogen Co. (Carlsbad, CA, USA). Fetal bovine serum (FBS) was obtained from Equitech-Bio, Inc. (TX, USA), and phosphate-buffered saline (PBS) from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). Enzyme-linked immunosorbent assay (ELISA) kits for the insulin assay were purchased from Shibayagi Co., Ltd. (Gunma, Japan). Tissue-Tek was purchased from Sakura Fine Technical Co. Ltd. (Tokyo, Japan). Goat normal serum and polyclonal guinea pig anti-insulin serum were purchased from Dako (Glostrup, Denmark). The 10% formalin solution, dithiothreitol (DTT) and Triton X-100 was purchased from Wako Pure Chemical (Osaka, Japan). NAP-5 column was purchased from GE health care (GE Healthcare UK Ltd., Buckinghamshire, UK).
Synthesis of polyDNA-PEG-phospholipid conjugate (polyDNA-PEG-lipid)

Mal-PEG-Lipid was synthesized by first dissolving NHS-PEG-Mal (180 mg), triethylamine (50 μL) and DPPE (20 mg) in dichloromethane and stirring for 36 h at room temperature as shown in Scheme 1 [12]. After precipitation with diethyl ether, Mal-PEG-lipid was obtained as a white powder (190 mg, yield 80%). $^1$H-NMR (CDCl$_3$, 400 MHz, $\delta$ ppm): 0.88 (t, 6H, -CH$_3$), 1.25 (br, 56H, -CH$_2$-) 3.64 (br, 480H, PEG), 6.71 (s, 2H, -HC=CH-, maleimide).

The structure of polyDNA-PEG-lipid was shown in Scheme 1. PolyA20 and polyT20 which carry (CH$_2$)$_6$-SS-(CH$_2$)$_6$-OH at 5’ end were purchased from Sigma-Aldrich Chemical Co. PolyDNA-SH was prepared by reduction of the disulfide bond with DTT in accordance with the instructions of the manufacturer. Briefly, polyDNA-disulfide conjugate (in 10mM Tris-HCl, 1mM EDTA pH 8.0) was mixed with DTT (0.04 M) for 16 h at rt for removal of protection group for thiol. After purification with NAP-5 column, polyDNA-SH was obtained.

The SH groups at the 5’-ends of polyDNAs were used to form conjugates with the Mal-PEG-lipid. A PBS solution of polyDNA-SH (1.0 mg) was mixed with Mal-PEG-lipid (5.0 mg) and the reaction mixture was left for 24 h at rt to form conjugations. polyDNA-PEG-lipid (500 μg/mL in PBS) was used for surface modification of cells and islets without any further purification.
Scheme 1. Synthetic scheme of polyDNA-PEG-DPPE. (a) Polyadenine (PolyA) and
polythymine (polyT) which carry (CH$_2$)$_6$-SS-(CH$_2$)$_6$-OH at 5’ end were obtained from Sigma-Aldrich Chemical Co. PolyA-SH and polyT-SH were prepared by reduction of disulfide by treatment with DTT. The polyA and poly T carry a thiol group at 5’ end. (b) Synthetic scheme of Mal-PEG-DPPE and conjugation with polyDNA-SH to prepare polyDNA-PEG-DPPE. (b) Schematic illustration of interaction between polyDNA-PEG-lipid and a lipid bilayer of the cell membrane, and immobilization of living cells to the islet surface. DNA (polyA20 or polyT20)-PEG-lipid has hydrophobic acyl chains which anchor into the cell membrane. After mixing polyT-PEG-lipid-modified GFP-HEK cells and polyA-PEG-lipid-modified islets, they were suspended in medium and cultured at 37 °C and 5% CO$_2$. During culture, GFP-HEK cells spread and grew on the islet surface.

**Encapsulation of islets with GFP-HEK cells**

HEK293, which stably expressed enhanced green fluorescence protein (EGFP) (GFP-HEK), were kindly supplied by Dr. K. Kato (Institute for Frontier Medical Sciences, Kyoto University). The GFP-HEK cells were routinely maintained in MEM supplemented with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. GFP-HEK cells were collected by centrifugation (180 × g, 5 min, rt) after treatment with trypsin. The cells were washed with HBSS to remove the medium. A cell pellet (2 × 10$^6$ cells) was obtained by centrifugation. After the addition of polyT20-PEG-lipid solution (50 µL, 500 µg/mL) to the cell suspension, the suspension was incubated for 1 hr with gentle agitation at rt. The cells were then suspended in 10 mL HBSS and collected by centrifugation (180 × g, 5 min, 25 °C, twice) to obtain polyT20-PEG-lipid-modified cells.

Islets were isolated from the pancreas of female Syrian hamsters (7-8 weeks old, Japan SLC, Inc., Shizuoka, Japan) using the collagenase digestion method. The islets were cultured for 7 d after isolation to remove cells damaged by the isolation procedure. The islets were maintained in culture medium (Medium 199 with 10% FBS, 8.8 mM
HEPES buffer, 100 units/mL penicillin, 100 μg/mL streptomycin, and 8.8 U/mL heparin). A solution of polyA20-PEG-lipid solution (500 μg/mL, 100 μL of PBS) was added to suspension of the islets in serum-free medium (200 islets), and the mixture was incubated at rt for 1 h. After washing three times with serum-free medium, polyA20-PEG-lipid-modified islets were obtained. Finally, polyT20-PEG-lipid-modified GFP-HEK cells (2 × 10^6) and polyA20-PEG-lipid-modified islets (200 islets) were mixed in serum free medium (300 μL), and the mixture was incubated for 60 min with gentle agitation at rt. The GFP-HEK cells-immobilized islets were picked up by hand using a Pasteur pipette (inside diameter: 1mm) under a stereo microscope and cultured on a non-treated dish at 37 °C under 5% CO₂ in Medium 199 supplemented with 10% FBS. Islets were observed by a confocal laser scanning microscope (FLUOVIEW, FV500, Olympus Optical Co. Ltd., Tokyo, Japan) and a phase-contrast microscope (IX71, Olympus). The FITC and GFP were excited by an argon laser (488 nm) and the fluorescence was detected through a bandpass filter (510 – 550 nm).

**Histochemical analysis**

GFP-HEK cells-immobilized islets were washed with PBS and then transferred, to 10% formalin solution and incubated for 1 d at rt. The formalin solution was removed and the islets were sequentially kept in 3% and 10% sucrose in PBS for 1 d, followed by incubation in 20% sucrose in PBS for an additional 1 d at rt. The islets were embedded in Tissue-Tek for freezing. The frozen specimens were sliced (6-μm thick) using a cryostat (CM 3050S IV, Leica, Solms, Germany). The sliced sections were permeabilized by treatment with 0.2% Triton X-100 in PBS at rt for 15 min. The samples were first treated with a 10% normal goat serum in PBS for 1 h to block the non-specific binding of
antibodies. The samples were then treated with 1% guinea pig anti-insulin serum in PBS containing 3% goat normal serum for 3.5 h at rt, and then washed with PBS. The samples were incubated with fluorescently-labeled secondary antibody, 0.2% Alexa 488 Goat anti-guinea pig IgG in PBS containing 3% goat normal serum, at rt for 1.5 h. Cell nuclei were counterstained with Hoechst 33342. The localization of secondary antibodies and the Hoechst dye was analyzed by a fluorescence upright microscope (BX51, Olympus). The sliced sections were also stained with hematoxylin-eosin (HE) using a conventional staining method.

**Insulin secretion from GFP-HEK cell-encapsulated islets on glucose stimulation**

Static insulin secretion tests were performed on GFP-HEK cells-immobilized islets (50 islets) after culturing for 3 days to evaluate their insulin-secreting ability in response to changes in glucose concentration. As a control experiment, this assay was performed on islets without enclosure with HEK cells (naïve islets). GFP-HEK cells-immobilized islets and naïve islets were exposed to solutions of glucose in Krebs-Ringer’s buffer (KRB) at concentrations of 0.1 g/dL, then 0.3 g/dL, and finally 0.1 g/dL glucose for intervals of 1 h in each solution at 37 °C. The supernatants were collected after each 1 h incubation and the insulin concentrations in the KRB solutions were determined by ELISA.

**Statistical analysis**

Comparisons between two groups were made using Student's *t*-tests. *p*<0.05 was considered statistically significant. All statistical calculations were performed using the software KaleidaGraph 4.0J.
RESULTS

Islets-encapsulation with living GFP-HEK cells

Islets were treated with polyA20-PEG-lipid, followed by treatment with FITC-labeled polyT20 (Figure 1(a)). Fluorescence from FITC-labeled polyT20 was clearly seen at the periphery of each islet (Figure 1(a-1)). No fluorescence was observed in naked islets treated with FITC-labeled polyT20 (Figure 1(a-2)). Thus, polyA20 can be immobilized onto the surface of islets using polyA20-PEG-lipid without damaging islet morphology and polyA20 on the cell surface can hybridize with polyT20.

GFP-HEK cells were immobilized onto the surface of islets by DNA hybridization between polyA20 on the islets and polyT20 on the GFP-HEK cells. Fig. 1(b) shows a microscopic image of a GFP-HEK cell-islet complex just after preparation. The HEK cells on the islet surface appeared as white cells under a phase contrast microscope (Figure 1, (b-2)) and were more clearly identified as green cells of GFP-HEK under a confocal laser scanning microscope (Figure 1, (b-1)). Single HEK cells were recognized on the islets, indicating that the GFP-HEK cells had become immobilized on the islet surface by DNA hybridization. The inhibition study was also performed. When polyA30-islets and polyT20-GFP-HEK were previously incubated with polyT20 and polyA20, respectively, no specific immobilization of GFP-HEK cells on islets was observed (Figure 1(d)).
Figure 1. Encapsulation of islets with living cells. (a) Hamster islets modified with polyA20-PEG-lipid were treated with (a-1, FITC) FITC-labeled polyT20. (a-2) Naked islets were treated with FITC-labeled polyT20. These islets were observed by a confocal laser scanning microscope. (b) Attachment of polyT20-PEG-lipid modified GFP-HEK cells onto the surface of polyA20-PEG-lipid modified hamster islets. An islet was observed by a confocal laser scanning microscope for (b-1, GFP) and a phase contrast microscope (b-2). (c) GFP-HEK cells-immobilized islets were cultured in Medium 199.
supplemented 10% FBS at 37 °C under 5% CO₂ for 1, 3, and 5 days. Islets were observed by a phase contrast microscope (left panels) and a confocal laser scanning microscope (right panels, GFP). Scale bars: 200 μm.

The modified islets were cultured in medium in a culture dish and observed at 1, 3, and 5 days after the complex was formed (Figure 1(c)). Although GFP-HEK cells were attached and spread on the surface after 1 day of culture, the islet surface was not completely covered with cells. The HEK cells spread and gradually proliferated on the islet surface, and by 3 days in culture, the islet surface was fully covered with a layer of HEK cells (Figure 1, (c)). As seen Fig. 1(c) and Fig. 2, HEK cells proliferated continuously and form a thicker multi cell layer due to lack of contact inhibition as the culture period proceeded.

**Histochemical analysis**

The GFP-HEK cell-encapsulated islets were histochemically analyzed by HE and insulin staining (Figure 2). In HE staining, a layer of GFP-HEK cells was observed around the islet surface after culturing for 3 and 5 days (left panel in Figure 2). The nuclei of GFP-HEK cells were slightly larger than that of islets. The multi-layers of GFP-HEK cells were formed on the islet surface after culturing for 3 and 5 days. Necrosis of islet cells was not observed even at the center of the complex at 5 days. Figure 2 also shows images of insulin-stained GFP-HEK cell-encapsulated islets (right panel). Islets stained green with anti-insulin antibody were found in a core cell aggregate. These results indicate that GFP-HEK cell-encapsulation did not impair the morphology of islets.
**Figure 2.** Histochemical analyses of GFP-HEK cells-immobilized islets cultured for 3 and 5 days. Frozen sections of GFP-HEK cells-immobilized islets were stained with (left panels) hematoxylin-eosin (HE) or (right panels) Alexa 488-labeled anti-insulin antibody and Hoechst 33342 dye for nuclear staining. Right panels show merged microscopic images of insulin (green fluorescence) and Hoechst 33342 staining (blue fluorescence). Scale bars: 100 μm.

**Insulin secretion by glucose stimulation**

We examined the abilities of the islets modified with or without HEK cell-encapsulation to release insulin. At the basal glucose concentration, 0.1 g/dL, insulin release by the encapsulated islets was less than that by naïve islets. We also performed a
glucose stimulation test to examine the ability of the modified islets to regulate insulin release in response to changes in the glucose level. When the glucose concentration in the medium was increased from 0.1 g/dL to 0.3 g/dL, islets of both groups increased insulin release above basal levels (Figure 3). Insulin release returned to basal levels when the islets were re-exposed to 0.1 g/dL glucose. Glucose stimulation indexes (insulin release at 0.3 g/dL glucose/insulin release at 0.1 g/dL glucose) calculated from these results were 7.3 ± 5.3 and 3.6 ± 1.0 for the encapsulated islets and naïve islets, respectively. Although there was no significant difference between two groups for glucose stimulation index, the

**Figure 3.** Glucose stimulation test of islets encapsulated HEK cells after 3 days culture. As a control experiment, the assay was performed on naive islets. The amounts of insulin secreted from the islets in response to glucose concentration changes (0.1, 0.3, 0.1 g/dL) were determined by ELISA. Results are expressed as mean ± SD for n = 3. KRB: Krebs-Ringer buffer. An asterisk represents a significant
amount of insulin secretion of the encapsulated islets tended to be lower than that of native islets at 0.1 g/dL and 0.3 g/dL glucose in KRB. These results indicate that the HEK cell layer did not influence the islets’ ability to regulate insulin release in response to glucose concentration, although the cause of decreased insulin release by encapsulated islets was not clear. It might be attributed to the oxygen consumption by the HEK293 cells since low oxygen tension can decrease islet cell function. The decrease of basal insulin release in encapsulated islets is a topic that requires further study.
DISCUSSION

There were some fundamental studies on improvement of biocompatibility by using cells such as chondrocyte [17, 18]. Pollok et al. first reported the macroencapsulation of rat islets with porcine chondrocyte membrane [17]. Porcine chondrocytes were cultured to form a confluent monolayer for use as a matrix upon which to deposit islets. After the islets were attached to poly(glycolic acid) polymer fibers, the islet-polymer composite was wrapped with chondrocyte membrane by hand. However, the increase of total volume after enclosure and aggregate formation of islets inside the membrane led to necrosis of islet cells at the center. Lee et al. also demonstrated enclosure of islets with chondrocyte membranes using the cell sheet engineering technique [18]. The issue of islet necrosis still remains to be resolved. They expected that chondrocyte membrane would work as an immuno-isolation membrane of bioartificial pancreas although they have not yet reported results of animal experiments unfortunately.

To overcome this problem, we studied methods to enclose islets singly with living cells. Herein, we have used amphiphilic polyDNA-PEG-lipid to immobilize GFP-HEK cells on the surface of islets through hybridization between polyA20 and polyT20. The surface of the islets was completely covered with a cell layer after 3 to 5 days in culture without central necrosis of the islet cells (Figure 2). Insulin secretion upon glucose stimulation was well maintained in the cells-encapsulated islets complex, although total insulin secretion was reduced as compared to normal islets (Figure 3). HEK cells which formed a multi cell layer on islets consume oxygen and thus islet cells
were exposed to low concentration of oxygen. We though that the reduction of insulin secretion was due to the insufficient oxygen supply to β-cells of islets. The same phenomena were observed in islets encapsulated with HEK cells using the biotin/streptavidin reaction. However, we might evade this issue by encapsulation with endothelial cells because they are expected to form a single cell layer.

We have reported immobilization of cells onto the islet surface by using biotin/streptavidin reaction [15]. Although the specific biotin/streptavidin reaction worked well to cover the islets with living cells, streptavidin is expected to activate the host immune system because it is a xenogeneic protein isolated from bacteria. Therefore we improved the technique compatible to future clinical application. There was no difference in islet encapsulation efficiency between these two techniques.

Many shortcomings remain to be overcome prior to clinical application. The cells employed are HEK293 cells which proliferate rapidly and form a multi-cellular layer. Most primary cells, that is, cells isolated from normal animals, proliferate much slower than HEK cells, and can not form a multi-cellular layer. Thus, we should select types of primary cells which can effectively form a cell layer on islets. The short and long term effects of the covered cells on islet functions should be carefully examined. We also should follow the fate of cells after transplantation. In addition, the efficiency and biocompatibility of cell-based microencapsulation should be examined carefully by using animal experiments early. Although these issues should be addressed, the technology developed in this study will be useful in preparation of future bioartificial pancreas and studies on cell-cell interaction.
REFERENCES


CHAPTER 5 Microencapsulation of islets with living cells


Chapter 1

The transplantation of islets of Langerhans has been successfully applied to the treatment of insulin-dependent diabetes. However, a shortage of human donors is the hardest obstacle to overcome. We aimed to develop a bioartificial pancreas that can realize xeno-islet transplantation. The islets were encapsulated in agarose microbeads carrying the soluble domain of human complement receptor 1 (sCR1), which is an effective inhibitor of the classical and alternative complement activation pathways. When naked rat islets were cultured in rabbit serum, large amounts of insulin leaked from the damaged islets over the course of a few days incubation, but no damaged cells were observed among islets in sCR1-agarose microbeads cultured in rabbit serum for 4 days. Although low levels of insulin were detected in the rabbit serum, the insulin did not leak from damaged β-cells, it was physiological insulin secreted by the β-cells.

Chapter 2

Transplantation of islets from xenogeneic sources such as porcine tissue has been proposed as potential solution to overcome the challenge of insufficient tissue supply. However, immunological reactions remained a major barrier to this approach. In previous study, we have developed novel bioartificial pancreas by incorporating the complement inhibitory effect of soluble complement receptor 1 (sCR1) into agarose-based microencapsulated islets. We have demonstrated in vitro that this technique could inhibit
complement activation and provide a better protection for islets from the destruction of xenoreactive antibodies and complement system in the serum as compared to non-treated microcapsules. Herein, we evaluate the in vivo performance of sCR1 conjugated agarose microencapsulated islets in a xenotransplantation model using streptozotocin (STZ) induced diabetic mice. Two groups of rat islets including agarose microencapsulated islets and sCR1-agarose microencapsulated islets were transplanted into intraperitoneal cavity of diabetic mice. No immunosuppression was used. In non-treated microcapsule groups, grafts were rapidly destroyed characterized by the reversal of blood glucose level to hyperglycemia. Graft survival was only 12.8 ± 4.2 days. In sCR1-agarose microcapsule groups, graft survival was markedly prolonged to more than 32 ± 10.7 days. Intraperitoneal glucose tolerance test (IPGTT) carried out at 30th day post-transplantation has revealed that mice transplanted with sCR1-agarose microencapsulated islets have similar glucose clearance profiles as normal non-diabetic mice, whereas mice transplanted with non-treated microcapsules remained hyperglycemia during the course of glucose stimulation. Islet encapsulated in sCR1-agarose membrane still remained viable at 31th day post-transplantation as indicated by histochemical staining for H&E and insulin.

Chapter 3

Transplantation of pancreatic islets of Langerhans (islets) is a promising method to treat insulin-dependent diabetes mellitus. Control of complement activation is necessary to improve graft survival in alloislet and xenoislet transplantation. In this chapter, human soluble complement receptor 1 (sCR1) was immobilized on the islet cell
surface through poly(ethylene glycol)-conjugated phospholipid (PEG-lipid) without loss of islet cell viability or insulin secretion ability. sCR1 on islets effectively inhibits complement activation and protects islets against attack by xenoreactive antibodies and complement. This method will be an efficient means to control early islet loss in clinical islet transplantation and realize xenoislet transplantation in the future.

Chapter 4

Early graft loss due to instant blood-mediated inflammatory reaction (IBMIR) is a major obstacle of clinical islet transplantation; inhibition of blood coagulation and complement activation is necessary to inhibit IBMIR. Here, human soluble form complement receptor 1 (sCR1) and heparin were co-immobilized onto the surfaces of islet cells. sCR1 molecules carrying thiol groups were immobilized through maleimide-poly(ethylene glycol)–phospholipids anchored in the lipid bilayers of islet cells. Heparin was immobilized on the sCR1 layer via the affinity between sCR1 and heparin, and additional layers of sCR1 and heparin were formed layer-by-layer. The sCR1 and heparin molecules in these layers maintained anti-complement activation and anti-coagulation activities, respectively. This promising method could be employed to reduce the number of islet cells required to reverse hyperglycemia and prolong graft survival in both allo- and xeno-islet transplantation.

Chapter 5

Microencapsulation of islets with a semipermeable membrane, i.e., bioartificial pancreas, is a promising way to transplant islets without the need for immuno-
suppressive therapy for insulin-dependent diabetes mellitus (type I diabetes). However, materials comprising a bioartificial pancreas are not ideal and might activate defense reactions against foreign materials. In this chapter, we propose an original method for microencapsulation of islets with living cells using an amphiphilic poly(ethylene glycol)-conjugated phospholipid derivative (PEG-lipid) and DNA hybridization. PolyA and polyT were introduced onto the surfaces of the islets and HEK 293 cells, respectively, using amphiphilic PEG-lipid derivatives. PolyA20 modified HEK cells were immobilized onto the islet surface where polyT20-PEG-lipid was incorporated. The cells spread and proliferated on the islet surface, and the islet surface was completely encapsulated with a cell layer after culture. The encapsulated islets retained the ability to control insulin release in response to glucose concentration changes.
LIST OF PUBLICATIONS

CHAPTER 1

N. M. Luan, Y. Teramura, and H. Iwata.

Immobilization of the soluble domain of human complement receptor 1 (sCR1) on agarose-encapsulated islets for the prevention of complement activation.

Biomaterials, 31, 8847-8853 (2010)

CHAPTER 2

N. M. Luan, and H. Iwata.

Xenotransplantation of sCR1-conjugated agarose microencapsulated islets.

In preparation.

CHAPTER 3

N. M. Luan, Y. Teramura, and H. Iwata.

Immobilization of soluble complement receptor 1 on islets.

Biomaterials, 32: 4539-4545 (2011)

CHAPTER 4

N. M. Luan, Y. Teramura, and H. Iwata.

Layer-by-layer co-immobilization of soluble complement receptor 1 and heparin on islets.

Biomaterials, 32: 6487-6492 (2011)
CHAPTER 5

Y. Teramura, N. M. Luan, and H. Iwata.

Microencapsulation of islets with living cells using polyDNA-PEG-lipid conjugate.


International Conference

N. M. Luan, Y. Teramura, and H. Iwata.

A novel bioartificial pancreas for xenotransplantation.

ACKNOWLEDGEMENTS

The present research was carried out from 2008 to 2011 under continuous guidance of Dr. Hiroo Iwata, Professor of the Institute for Frontier Medical Sciences, Kyoto University. The author is deeply indebted to Professor Iwata for his constant guidance, encouragement, valuable discussion, and detailed criticism on the manuscript throughout the present work. The completion of the present research has been an exciting project and one which would not have been possible without his guidance.

The author wishes to express his special thanks to Dr. Yuji Teramura, Assistant Professor of the Institute for Frontier Medical Sciences, Kyoto University, for his thoughtful guidance, helpful suggestions, and intimate advice.

The author is also very grateful to Dr. Koichi Kato, Associate Professor of the Institute for Frontier Medical Sciences, Kyoto University, for his enriching suggestions and encouragement. The author also would like to express his thanks to Dr. Yusuke Arima, Dr. Satoshi Fujita, Dr. Mitsuaki Toda, Dr. Tadashi Nakaji-Hirabayashi, Dr. Tomonobu Kodama, Mr. Chen Hao, Mr. Shuhei Konagaya, Mr. Naohiro Takemoto and all the members of Professor Iwata’s laboratory, for their kind help and candid comments. The author also would like to express his thanks to Mr. Takatoshi Matsushita and Ms. Kumiko Kogishi for their kind help in histological examination. General acknowledgements are due to Ms. Yoshiko Suzuki.
The author would like to express his sincere appreciation to the Japan International Cooperation Agency (JICA) for their financial support. The author also would like to express his gratitude to Mrs. Miyuki Arita, Ms. Makiko Shimomura, Ms. Yoko Kinashi, Ms. Yuko Hikichi, Ms. Sachiko Namba and Ms. Maria Tsunosue, officers from JICE and JICA, for their constant care and their kind help throughout the period.

Finally, the author would like to take the opportunity to extend hearty thanks to his parents and his wife for their cordial support and continuous encouragement.

June, 2011
Kyoto, Japan.

Nguyen Minh Luan