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厚生労働科学研究費補助金
再生医療実用化研究事業

生着率の向上を目指した膵ランゲルハンス島の表面改質に関する研究

平成２０年度　総括研究報告書

研究代表者　寺村　裕治

平成２１（２００９）年　３月

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厚生労働科学研究費補助金（再生医療実用化研究事業）
（総括）研究報告

生着率の向上を目指した膵ランゲルハンス島の表面改質に関する研究

研究代表者　寺村裕治　京都大学　助教

研究要旨

I型糖尿病の根本的な治療法として、膵ランゲルハンス島の移植が注目されている。高い成功率での移植法が確立されたものの、免疫抑制剤による副作用や移植後の生着率に関しては問題点が残されている。本研究では、ポリエチレングリコール（PEG）結合脂質（PEG結合脂質）と高分子積層膜を利用して、膵島の表面修飾の取り組み、生着率の向上を目指している。PEG脂質で表面修飾した膵島を糖尿病マウスの肝臓内へ移植し、生着率の評価を行った。未処理の膵島の移植群と比較し、正常血糖値期間、移植直後に細胞障害を受けた膵島から放出されるインスリン量、組織化学的評価から、PEGによる表面修飾が生着率に与える影響を詳細に調べた。膵島表面にPEG修飾することで、移植直後に生じる凝固系や補体活性化に起因する膵島への細胞障害が著しく軽減できることが分かった。このことは、膵島表面でのみ生じる反応を抑制することで、生着率を向上できることを示す結果である。

A. 研究目的

本研究は、全く新しいバイオ人工膵臓を世界に先駆けて開発し、臨床での早期利用を目指したものである。これまでに開発されたバイオ人工膵臓の大きな問題点としては、(1)カプセル膜厚が大きいことと、(2)移植後に副作用（血栓症や炎症反応）が起きることがあげられており、臨床試験への大きな足かせとなっていた。この問題を解決するために、本研究では細胞表面にナノからマイクロレベルでの高分子薄膜を形成させ、表面修飾後の膵島の体積増加が起こらないようにした。膜内に生理活性物質（ヘパリン、ウロキナーゼ）を担持させ、抗血栓能や抗炎症能を有するものであり、移植後の生着率の向上を試みるものである。具体的には、同一分子内に親水性高分子と長鎖疎水部を有する両親媒性高分子（ポリエチレングリコール結合脂質）と積層膜を利用したものである。生着率を高める目的で生理活性物質を担持したバイオ人工膵臓の開発を行う。併せて、マウス糖尿病モデルを用いて、*in vivo* 評価を行う。本研究では、高分子薄膜により表面修飾した膵島の開発と移植後の炎症反応を抑制できる機能性素材の開発に取り組み、高い生着率が実現できるバイオ人工膵臓の開発を世界に先駆けて行う。

B. 研究方法

同一分子内に親水性高分子と長鎖疎水部を有する両親媒性高分子（ポリエチレングリコール（PEG）脂質）と積層膜を利用した薄膜に生理活性物質を固定した。表面修飾後の膵島の体積増加が殆ど起こらないため、臨床での膵島移植術がそのまま適用でき、移植した膵島の生着率が向上することが期待できる。*in vivo* 評価として、糖尿病マウスの肝臓内へバイオ人工膵臓の移植を行った。ストレプトゾトシンにて糖尿病を誘発したマウスへ、PEG脂質を用いて膵島表面（ハムスター由来）をPEG層によりカプセル化したバイオ人工膵臓を移植した。未処理の膵島群として、比較して実験を行った。移植後の血糖値測定、血中インスリン測定、肝臓のHE染色像から評価を行った。

（倫理面への配慮）

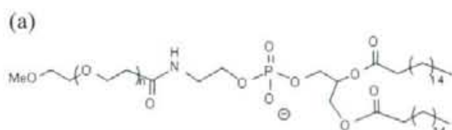
本研究で行う動物実験は、京都大学の動物委員会の承認を得た後、「京都大学における動物実験の実施に関する規程」に従って実験を行う。

C. 研究結果

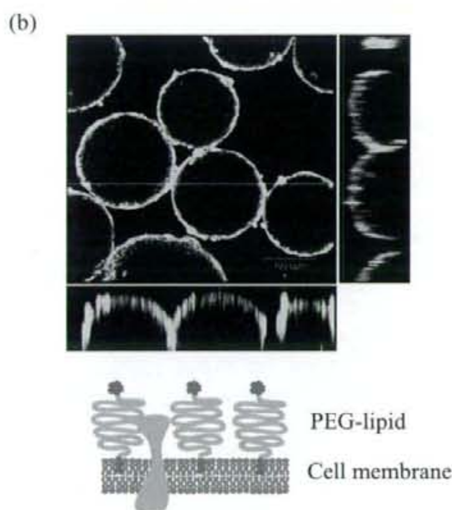
これまでに両親媒性高分子を用いて、膵

島や細胞の表面修飾を行ってきた。この方法は、細胞への障害が与えないし、体積増加が起きないから肝臓内への移植を考えた場合、非常に有用な手法になる。両親媒性高分子を用いて、膵島表面にポリエチレングリコール愁傷区や生理活性物質の固定化（血栓溶解酵素であるウロキナーゼの固定化）に取り組んできた。肝臓内へ移植した際の炎症反応や凝固反応が関与する膵島への細胞障害を軽減することを目指している。本実験では、表面修飾剤として、図1(a)に両親媒性のPEG結合脂質を使用した。

(a)



(b)



(c)

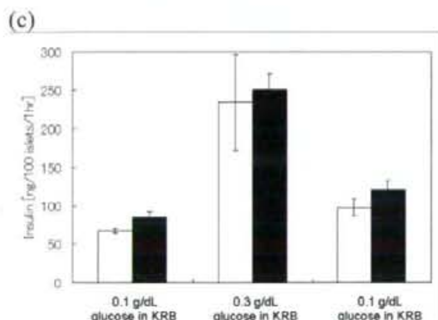


図 1. PEG 脂質による膵島の表面修飾
(a) PEG 脂質の化学構造式, (b) FITC 標識した PEG 脂質で表面を修飾した膵島の共焦点レーザー顕微鏡像(c) グルコース負荷試験 インスリンは、ELISA 法により決定した。

PEG 脂質を膵島分散液と混合することで、膵島表面の脂質二重層へその疎水性ドメインの脂質部分が自発的に導入される（図 1(b)）。共焦点顕微鏡像から分かるように、膵島表面にのみ蛍光がみられることから、膵島表面に PEG 脂質が導入されていることが分かる。このように、PEG 脂質を用いることで、膵島表面に PEG 層を形成することが可能になった。

また、グルコース負荷試験により、膵島のインスリン分泌能への影響を調べた（図 1(c))。正常の膵島では、低血糖値から高血糖値へと変化するにつれて、インスリン分泌量は増加し、再び血糖値を下げるとインスリン分泌量は低下する。PEG 脂質で表面修飾した膵島でも、同様のグルコース応答が認められ、正常に機能していることが分かった。

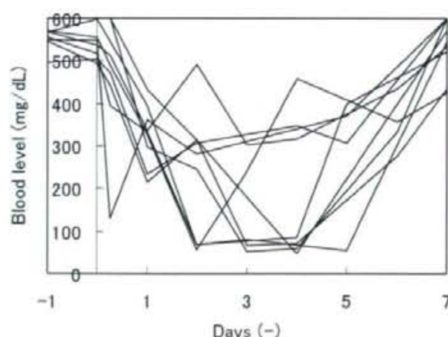
糖尿病マウスの肝臓内へ門脈を通して、ハムスター由来の膵島を移植した。この移植モデルは、急性拒絶がおきない（concordant）異種移植モデルとなっている。レシピエントマウスへの免疫抑制剤の投与おこなっていない。本実験では、PEG脂質による表面修飾の効果をしらべ、移植直後（数日間の）の膵島の生着率を評価することを目的としており、この異種移植のモデルを選択した。PEG脂質で修飾した膵島あるいは膵島を肝臓内へ500個移植した後の血糖値変化を図2(a)と(b)にそれぞれ示す。異種移植モデルなので、ハムスター膵島を糖尿病のマウスへ移植した後、およそ一週間後には拒絶反応によりグラフトが消失し、正常血糖値が維持できず、高血糖状態へ戻る。膵島を500個移植した場合、移植一日後の血糖値は、ほとんどのマウスにおいて、正常血糖値まで戻らず、3日後から正常血糖値を示している。半数のマウスでは、正常血糖値を示さず、300mg/dL付近まで低下した後、一週間後に高血糖状態へ戻るマウスもみられた。このことは、肝臓内への移植

直後に膵島への細胞障害がおき、残存する膵島では、高血糖状態の血糖値を正常に制御できないものと考えている。

他方、PEG-膵島を500個移植した場合では、移植一日後では、半数のマウスでは、正常血糖値を示さないものの、2日後以降では、ほとんどすべてのマウスが正常血糖値を維持した。500個の膵島あるいはPEG-膵島を移植した場合について、グラフトの生存期間を比較した。表1にその結果を示す。グラフトの生存期間は、両グループにおいて有意な差がみられた。このことから、移植直後の膵島への細胞障害は、PEG修飾により抑制できたために、免疫拒絶反応によるグラフト消失までの生存期間が延長したものと考えられた。

以上のことから、PEG修飾により、移植直後の補体や血小板凝集が関与する細胞障害は抑制できるものの、免疫拒絶反応は抑制できないことが分かった。

(a)



(b)

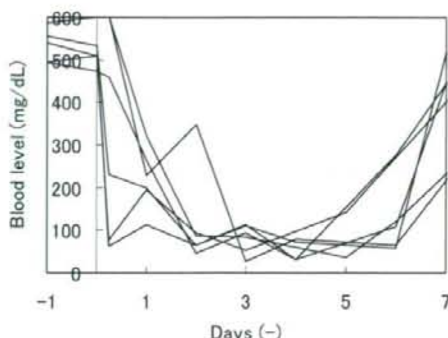


図 2. 膵島移植後の糖尿病マウスの血糖値変化 (a)未処理の膵島（500 islets），(b) PEG 脂質で表面を修飾した膵島（500 islets）

PEG-膵島あるいは膵島を、門脈を通して肝臓内へ移植した後、1 時間あるいは 1 日後の肝臓の組織学検査（HE 染色）をいった（図3）。図 3(a)で示すように膵島移植後 1 時間後では、肝臓内の血管内に存在している膵島の崩壊が認められた。崩壊した膵島の周辺には、赤血球の凝集塊がおおく存在しており、表面で血小板凝集や血液の凝固が起きていることが示唆される。また、一日後でも同様に膵島への障害が見られている。また、血管内壁が障害を受けていることが分かる。

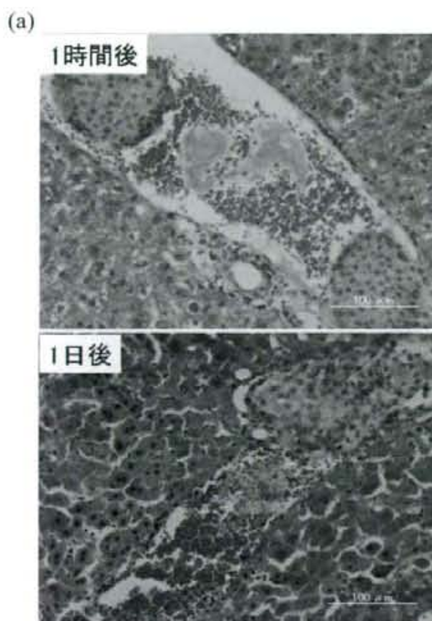
Table 1. Graft survival days after transplantation (500 islets)

	Graft survival days	Mean±SD
control islets	0, 0, 0, 1, 2, 3, 3, 4	1.8±1.6
PEG-islets	4, 4, 5, 5, 6, 6	5.0±0.9

Graft failure was defined as two consecutive plasma glucose determinations > 200 mg/dL.

しかしながら、図3(b)で示したようにPEG修飾した膵島では、移植後1時間後では、膵島への障害はほとんどみられず、肝臓内にとどまっている。また、一日後でも同様のことが見られている。従って、未処理の膵島では、細胞障害を受けて崩壊しているが、PEG修飾により移植直後の膵島表面上の血中成分との挙動は、無処理の膵島表面上のそれとは、異なった。

(a)



(b)

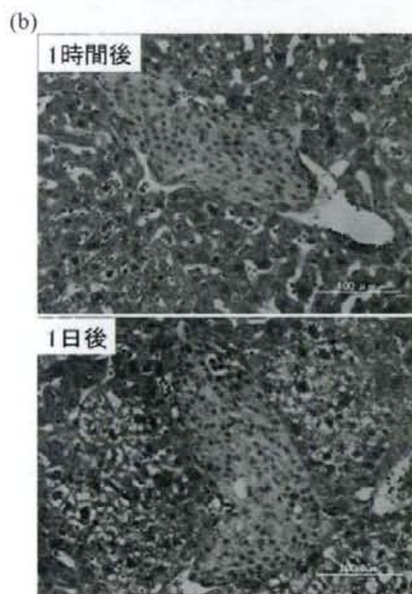


図 3. 膵島移植後の糖尿病マウスの肝臓の HE 染色像 (a)未処理の膵島移植後 1 時間後あるいは 1 日後（500 islets）, (b) PEG 脂質で表面を修飾した膵島移植後 1 時間後あるいは 1 日後（500 islets）

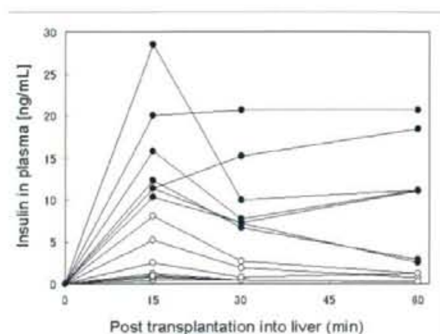


図 4. 膵島移植後のマウス血中インスリン濃度変化. ●: 未処理の膵島移植群(1000 islets), ○: PEG 脂質で表面修飾した膵島移植群(1000 islets).

膵島あるいはPEG-膵島を肝臓内へ移植した後、15,30,60分後に採血を行い、血中のインスリンを定量した（図4）。細胞障害を受けた膵島が崩壊すると、その中に存在しているインスリンの放出がみられ、血中のインスリン濃度が一時的に増加する。そのために、血中インスリン濃度を測定することで、膵島への細胞障害の程度を判断できるパラメーターになる。未処理の膵島を移植すると、移植後15分後には大量のインスリンが放出された。また、その放出量の減少がみられるものの、30、60分後も放出が見られた。これは、移植直後に膵島が細胞障害を受けて崩壊し、大量のインスリンが放出されたものと考えられる。他方、PEG-膵島を移植した場合では、移植直後のインスリンの放出は著しく抑制された。このことは、PEG修飾により膵島への細胞障害が抑制されてものと考えられる。

このことより、PEG 修飾により、移植直後の血液適合性を著しく向上できることを示すことができた。

D. 考察

両親媒性のPEG脂質を用いて膵島表面を修飾し、肝臓内への移植した後の生着率について検討した。PEG脂質は、細胞毒性がなく疎水性相互作用により自発的に脂質二重層へ導入される。従って、膵島表面にPEG層を形成することが可能になり、体積変化

のない表面修飾が実現できる。

PEG脂質は、これまでにDDSの分野にて、リポソームの表面修飾に利用されてきた。リポソームをPEG脂質にて、表面修飾することで血中滞留時間が著しく延長することが明らかにされている。PEG修飾したリポソームの表面上では、補体活性化の抑制や血小板凝集の抑制が報告されている。我々のグループでは、SPR法により、PEG修飾した基板上での補体の活性化を調べている。PEG修飾基板では、C3bの吸着は抑制されることから、補体の活性化はみられない。

本実験では、PEG脂質にて表面修飾したハムスター膵島を糖尿病マウスへ移植した。免疫拒絶反応によりグラフトが消失するまでの生着期間について、未処理の膵島とPEG脂質で修飾した膵島を比較している。PEG修飾した膵島を移植した場合では、グラフト生存期間が有意に延長することがわかった。また、移植直後の血中のインスリンレベルから、PEG修飾することで、膵島からのインスリン放出が抑制でき、細胞障害は著しく抑制されていることがわかった。PEG修飾した膵島表面でも、補体活性化の抑制、血小板凝集の抑制がおきていることが考えられる。

E. 結論

膵島表面に PEG 修飾することで、移植直後に生じる凝固系や補体活性化に起因する膵島への細胞障害が著しく軽減できた。このことは、PEG 鎖がこれらの反応を抑制していることを示しており、膵島表面でのみ生じる反応を抑制することで、生着率を向上できることを示す結果である。

F. 健康危険情報

特に報告事項はなし。

G. 研究発表

1. 論文発表

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(2) Carlos A Agudelo, Yuji Teramura, and Hiroo Iwata, "Cryopreserved Agarose-Encapsulated Islets as Bioartificial Pancreas: a feasibility study" *Transplantation*, **87**, 29-34 (2009).

(3) Yuji Teramura and Hiroo Iwata, "Islets of Langerhans encapsulated with a layer of living cells for improvement of biocompatibility" *Biomaterials,* **30**, 2270-2275 (2009).

2. 学会発表

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(7) 寺村裕治、岩田博夫、「ポリエチレングリコールにより表面修飾した膵島の生着率評価」第 36 回膵・膵島移植研究会 (2 月 28 日、福岡)

H. 知的財産権の出願・登録状況

特になし

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yuji Teramura Hiroo Iwata	Islets surface modification prevents blood-mediated inflammatory responses	*Bioconjugate Chem*	19	1389-1395	2008
Carlos Agudelo, Yuji Teramura, Hiroo Iwata	Cryopreserved Agarose- Encapsulated Islets as Bioartificial Pancreas: a feasibility study	*Transplantation*	87	29-34	2009
Yuji Teramura Hiroo Iwata	Islets of Langerhans encapsulated with a layer of living cells for improvement of biocompatibility	*Biomaterials*	30	2270-2275	2009

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Islets Surface Modification Prevents Blood-Mediated Inflammatory Responses

Yuji Teramura, and Hiroo Iwata

Bioconjugate Chem., **2008**, 19 (7), 1389-1395• DOI: 10.1021/bc800064t • Publication Date (Web): 06 June 2008

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Islets Surface Modification Prevents Blood-Mediated Inflammatory Responses

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Transplantation of islets of Langerhans (islets) is a promising technique for treating insulin-dependent diabetes mellitus (type I). One unresolved issue is early graft loss due to inflammation triggered by blood coagulating on the surface of islets after transplantation into the portal vein. Here, we describe a versatile method for modifying the surface of islets with an ultrathin membrane carrying the fibrinolytic enzyme urokinase or the anticoagulant heparin. The surface of islets was modified with a poly(ethylene glycol)−phospholipid conjugate bearing a biotin group (biotin-PEG-lipids, PEG MW: 5000). Biotin-PEG-lipids were anchored to the cell membranes of islets, and the PEG-lipid layer on the islets was further covered by streptavidin and biotin−bovine serum albumin conjugate using a layer-by-layer method. The surface was further activated with oxidized dextran. Urokinase was anchored to the islets through Schiff base formation. Heparin was anchored to the islets through polyion complex formation between anionic heparin and a cationic protamine coating on the islets. No practical islet volume increase was observed after surface modification, and the modifications did not impair insulin release in response to glucose stimulation. The anchored urokinase retained high fibrinolytic activity, which could help to improve graft survival by preventing thrombosis on the islet surface.

INTRODUCTION

The success achieved with the Edmonton protocol has established clinical islet transplantation as an alternative to pancreas transplantation (*1, 2*). Transplantation of islets of Langerhans (islets) has been proposed as a safe and effective method for treating patients with insulin-dependent diabetes mellitus (type I). However, it still remains an experimental procedure for late-stage diabetics who can no longer control diabetes with insulin injection. Some improvements in islet transplantation are needed, including increasing islet isolation efficiency, improving islet preservation, increasing the efficacy of immunosuppressive drug dosage protocols, and reducing islet loss in the early phase following transplantation. Among these, graft loss poses the most serious limitations for current islet transplantation protocols. Even though sufficient numbers of islets from multiple donors are transplanted to one patient, only 30% of patients treated become insulin-independent (*2*), suggesting that many islets are lost early after intraportal transplantation.

Innate immune reactions are involved in the destruction of islets exposed to blood in the portal vein (*3–6*). The blood coagulation and complement systems are activated when islets are exposed to fresh blood in the portal vein. Chemotactic factors, tissue factor, chemokines, and other inflammatory mediators are released during this early phase. These factors induce instant blood-mediated inflammatory reactions that result in graft loss (*7*). Some methods of regulating early coagulation and blood-mediated inflammatory reactions have been investigated, such as administering the thrombin inhibitor Melagatran (*8*), activated protein C (*9*), dextran sulfate (*10*), and the water-soluble domain of complement receptor I (*11*). Control of these reactions has promoted the transplantation success by reducing islet loss in animal models; however, it is difficult to apply these methods in the clinical environment because systemic administration is associated with an increased risk of bleeding. Recently, Nilsson et al. proposed an original method for preventing instant blood-mediated inflammatory reactions by coating islets with heparin to inhibit surface thrombosis formation following transplantation (*12*). Although it is a challenging method to reduce islet loss in the early phase of transplantation, it has been experimentally under examination. At this point, it is valuable to propose several alternative methods to modify islet surface.

In our previous studies (*13, 14*), islets were enclosed in a thin synthetic polymer membrane through poly(ethylene glycol)-conjugated phospholipid (PEG−lipids) derivatives that had been anchored in the membranes of islets cells. The PEG−lipid derivatives can be immobilized on the cell surface through hydrophobic interactions between the lipid bilayer and the PEG−lipid, without any effect on cell viability or function. The polymer membrane can be covered further using the layer-by-layer method (*13, 14*). In this report, we propose a versatile method for immobilizing enzyme and glycosaminoglycans, such as urokinase and heparin, to the surface of islets utilizing this layer-by-layer method. In addition, the results of detailed studies on the fibrinolytic properties of islet-anchored urokinase are reported.

EXPERIMENTAL PROCEDURES

Materials. α-*N*-Hydroxysuccinimidyl-*ω*-*tert*-butoxycarbonyl poly(ethylene glycol) (NHS-PEG-Boc, MW 5000) was purchased from Nektar Therapeutics (San Carlos, CA). 1,2-Dipalmitoyl-*sn*-glycerol-3-phosphatidylethanolamine (DPPE) was purchased from NOF Corporation (Tokyo, Japan). Dichloromethane, chloroform, *N,N*-dimethylformamide (DMF), diethyl ether, *N,N'*-dicyclohexylcarbodiimide (DCC), D-biotin, streptavidin from Streptomyces avidin, dextran (MW 200 000−300 000), protamine sulfate (from Salmon), and heparin sodium salt were purchased from Nacalai Tesque (Kyoto, Japan). Sodium perio-

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date ($NaIO_4$) was purchased from Wako Pure Chemcal (Osaka, Japan). Fluorescein isothiocyanate (FITC) was purchased from Dojindo Laboratories (Kumamoto, Japan). Urokinase was purchased from Calbiochem Inc. (La Jolla, CA). Biotinylated bovine serum albumin (biotin-BSA, biotin/BSA = 9, by molar ratio) was purchased from Pierce Biotechnology Inc. (Rockford, IL). Fibrinogen (human) and FITC-labeled BSA (FITC-BSA) were purchased from Sigma-Aldrich Co. (St Louis, MO). Thrombin was purchased from Kayaku CO. Ltd. (Saitama, Japan). FITC-streptavidin was purchased from Zymed laboratories (South San Francisco, CA). Biotinylated poly(ethylene glycol) alkanethiol was purchased from SensoPath Technologies Inc. (Bozeman, MT). Minimum essential medium (MEM), HEPES buffer solution, and Medium 199 were purchased from Invitrogen Co. (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from BioWest (Miami, FL). Phosphate-buffered saline (PBS) was purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan).

Synthesis of Biotinylated PEG-Conjugated DPPE. NH_2−PEG−lipid was synthesized from DPPE and NHS-PEG-Boc, which carries an activated ester (NHS) to react with an amino group of DPPE, and a protective group (Boc) for the amino group, as reported previously (*13*). Briefly, NHS-PEG-Boc (175 mg) and DPPE (21 mg) were dissolved in 5 mL of dichloromethane solution, and stirred for 3 days at room temperature (RT). Then, a solution of TFA (2 mL) was added and stirred for 20 min at 4 °C to remove Boc groups. The crude product was purified by precipitation with diethyl ether. After extraction into chloroform and evaporation, NH_2−PEG−lipid was obtained as a white solid (115 mg, yield 66%). D-Biotin (20 mg) and DCC (21 mg) were dissolved into DMF and stirred for 3 h at RT. Then, NH_2−PEG−lipid (22 mg) was added to the solution and stirred for 7 days at RT. This solution was filtered through a glass filter and the DMF was evaporated *in vacuo*; then, chloroform was added. After precipitation with diethyl ether, biotin-PEG-lipid was obtained as a white powder (10 mg, yield 40%). Biotin-PEG-lipid: ^{1}H NMR ($CDCl_3$, 400 MHz, δ ppm): 0.88 (t, 6H, -CH_3), 1.25 (br, 52H, -CH_2-, DPPE), 3.18 (d, 2H, C-CH_2-S, biotin), 3.24 (q, 1H, S-CH(-C)-C, biotin), 3.64 (br, 460H, PEG). 4.52 (m, 2H, C-CH(-C)-N, biotin), 5.15 (s, 2H, C-NH-C, biotin).

Forming Biotin-BSA and Streptavidin-Based Multilayers. A multilayered protein membrane was formed with biotin-BSA and streptavidin using the layer-by-layer method. To examine the formation of the multilayered membrane, we employed an in-house-designed surface plasmon resonance (SPR) instrument (*15*). Gold-coated BK-7 glass plates were immersed in a biotin-SAM (1 mM) methanol solution for 24 h, and the biotin-SAM formed on the surface of the gold-coated glass plates. Before SPR measurements, the plates were sequentially washed thoroughly with pure water and 2-propanol. A glass plate was set in an SPR flow cell, and a solution of strepavidin (20 μg/mL in PBS) was allowed to flow onto the surface, followed by a solution of biotin-BSA (40 μg/mL in PBS). These procedures were repeated several times to form multiple layers of BSA and streptavidin by the layer-by-layer method. The reaction solutions and PBS were allowed to circulate through the flow cell at 4.0 mL/min, and all SPR measurements were performed at 30 °C.

Modifying a Single Cell Surface with Biotin-PEG-Lipid and Streptavidin Complex. CCRF-CEM cells (acute lymphoblastic leukemia T-cells) were used as model cells for the surface modification of single cells. Biotin-PEG-lipids were dispersed in MEM and added to a CCRF-CEM cell suspension (1×10^6 cells in MEM, [biotin-PEG-lipids] = 100 μM), the cell suspension was incubated for 30 min at RT. The cells were washed with MEM and centrifuged at $180 \times g$ for 5 min at RT. This procedure was repeated twice. The biotin-PEG-modified cells were resuspended in 80 μL of MEM. Then, 20 μL of FITC-labeled streptavidin was added to the cell suspension (100 μg/mL) and incubated at RT for 5 min. Cell viability was assessed by the Trypan blue exclusion method. FITC-labeled streptavidin-modified cells were observed by confocal laser scanning microscopy (FLUOVIEW FV500, Olympus, Tokyo).

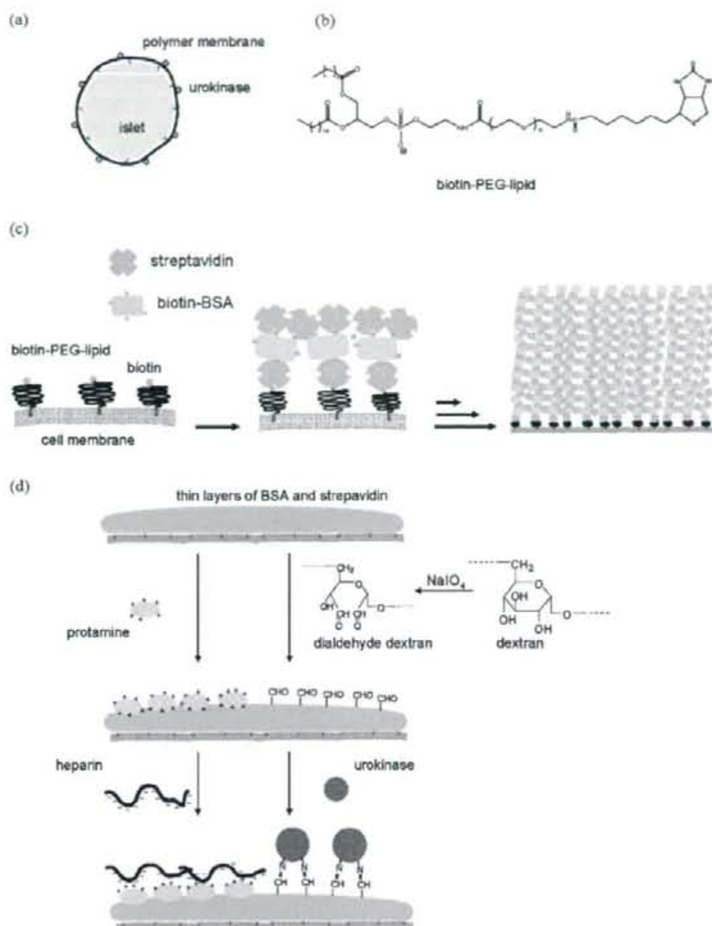
Islets Surface Modification. Islets were isolated from the pancreas of Syrian hamsters (7−8 weeks old, female, Japan SLC, Inc., Shizuoka, Japan) by the collagenase digestion method (*24*). The islets were maintained in culture medium (Medium 199 with 10% FBS, 8.8 mM HEPES buffer, 100 units/mL penicillin, 100 μg/mL streptomycin, and 8.8 U/mL heparin). A large amount release of insulin was observed from damaged islets during a few days culture after the islet isolation. Isolated islets were cultured for 7 days to remove damaged cells before surface modification. Effect of surface modification on islet functions could be clearly seen after this 7 days culture. A biotin-PEG-lipid solution was added to islets suspended in MEM (100 islets, [biotin-PEG-lipid] = 500 μM 100 μL of MEM), and the mixture was incubated at RT for 1.5 h. After washing three times with MEM, the biotin-PEG-lipid modified islets were mixed with a solution of streptavidin (50 μg/mL in PBS) and allowed to react for 10 min at RT. Streptavidin-modified islets were washed three times with MEM. The modified islets were then added to a solution of biotin-BSA (100 μg/mL in PBS) and left for 10 min at RT. The layer-by-layer modification with streptavidin and biotin-BSA was repeated 10 times to form 20 layers on the surface of the islets. In order to visualize the surface of islets by confocal laser scanning microscopy), FITC-labeled streptavidin (30 μg/mL in PBS) was used for the 21st layer.

Anchoring Bioactive Molecules onto the Surface-Modified Islets. The surface-modified islets were further modified with the fibrinolysis enzyme urokinase or with heparin, using dialdehyde dextran (*16*) or protamine. $NaIO_4$ (107 mg) was added to a dextran (250 mg) solution in pure water (5 mL) and the reaction mixture was stirred for 2 h at RT. Oxidation of dextran by $NaIO_4$ causes cleavage of the carbon−carbon bonds within each monosaccharide unit of the dextran chain, transforming the 1−2 hydroxyl groups into dialdehyde groups. After precipitation with acetone and dialysis against PBS, dialdehyde dextran was obtained as a white solid (220 mg). Biotin-BSA/streptavidin-modified (20 layers) islets were then activated using the dialdehyde dextran solution (5 mg/mL in PBS) for 30 min at 37 °C, and then incubated in urokinase solution (1000 IU/mL in PBS) for 60 min at 37 °C to anchor the urokinase onto the islets. Urokinase-islets were cultured in Medium 199 supplemented with FBS, heparin, and HEPES for a specified number of days at 37 °C in 5% CO_2.

Fibrinolytic activity of the anchored urokinase was determined by the fibrin plate method (*17*). Briefly, 10 mL of a fibrinogen solution (10 mg/mL in saline) (ϕ = 9.5 cm) and 60 μL of a thrombin solution (60 IU/mL) was mixed well. Plasminogen was not added to the fibrinogen solution, because plasminogen is included in fibrinogen prepared by the standard preparation (*17*). Naïve islets and urokinase-anchored islets (50 islets) collected after 0, 1, 3, and 7 days of culture were spotted onto the fibrin plate and incubated at 37 °C for 12 h. The size of the area of dissolved fibrin around the islet spot was determined as a measure of urokinase fibrinolytic activity.

For anchoring heparin, the BSA and streptavidin-modified islets (total layers, 10) were further treated with polycation, protamine (150 μg/mL in HBSS), for 5 min at RT. Most of polycations are cytotoxic toward many cell types. In this study, protamine was used as a polycation, because it is the least cytotoxic polycation as could be expected from the fact that it

Scheme 1[a]



[a] (a) Scheme for anchoring urokinase on surface of islets by constructing a multilayered membrane composed of streptavidin and biotin-BSA. (b) Chemical structure of biotin-PEG-conjugated DPPE (biotin-PEG-lipid). (c) Biotin-BSA/streptavidin multilayered membrane formation. The first biotin-PEG layer was anchored to the surface through hydrophobic interactions. Then, streptavidin and biotin-BSA were chemically reacted to the surface, layer-by-layer. (d) Dextran was oxidized to produce aldehyde groups on the surface of islets, providing a means for urokinase immobilization through Schiff base formation. Heparin was anchored to the surface of islets after treatment with protamine.

is administered to reverse heparin action in the clinical setting. Then, heparin was added for 15 min at RT (5 mg/mL in HBSS). For visualization, the heparin had been labeled with FITC. Heparin (40 mg/mL in pure water) was reacted with FITC (2.6 mg in 100 μL of DMSO) for 3 h at RT, precipitated in acetone at -20 °C for 24 h, evaporated, and washed three times with acetone. Finally, the precipitate was dialyzed against PBS for 2 days (MWCO; 3500 Da) to obtain FITC-heparin (FITC/heparin = 2.5 by molar ratio).

Glucose Stimulation Test. A static glucose-responsive insulin assay (*13*) was performed on biotin-BSA/streptavidin-encapsulated islets, activated dextran modified-encapsulated islets, and urokinase-immobilized islets after 1 and 7 days in culture. After the modified islets were washed several times with Krebs-Ringer solution, they were incubated in 0.1 g/dL, 0.3 g/dL, and then 0.1 g/dL glucose in Krebs-Ringer solution for 1 h each at 37 °C. The insulin concentration in the supernatant at each step was determined by an enzyme-linked immunosorbent assay (ELISA; Shibayagi Co. Ltd., Gunma, Japan).

Statistical Analyses. Comparisons between two groups were performed by Student's t test. $P < 0.05$ was considered statistically significant. All statistical calculations were performed using the software *JMP v.5.1.1*.

RESULTS

Forming Multilayered Biotin-BSA and Streptavidin Membranes on Surface of Islets. A nanometer-thick membrane composed of streptavidin and biotin-BSA layers formed on cell surfaces using biotin-PEG-lipid and the layer-by-layer method, as shown in Scheme 1. Biotin and streptavidin interact to form a stable conjugate (association constant was $\sim 10^{15}$ M^{-1}) under physiological conditions, so this system can be used to form a multilayered membrane without damaging the surface of the living cell. To confirm the formation of a multilayered biotin-BSA and streptavidin membrane, the SPR method, which can detect small changes in refractive index on the surface of a gold thin film in real time, was employed. Figure 1 shows the SPR profiles of layer-by-layer reactions between streptavidin and

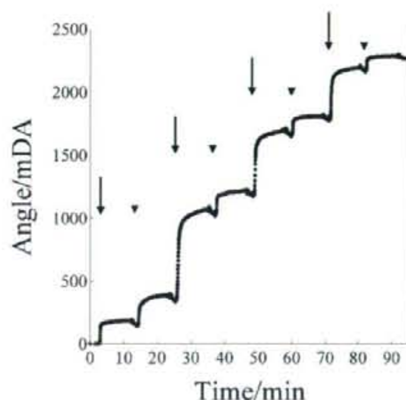


Figure 1. SPR-monitoring of multilayered streptavidin and biotin-BSA membrane formation on the biotin-SAM surface. Stepwise SPR profile indicates sequential formation of a protein multilayer. Arrows and arrowheads indicate the addition of streptavidin and biotin-BSA solutions, respectively.

biotin-BSA. A solution of streptavidin was flowed onto an SPR sensor with a self-assembled monolayer of biotinylated poly-(ethylene glycol) alkanethiol (biotin-SAM) as indicated by the first arrow in Figure 1 and Supporting Information Figure S1. The streptavidin layer was formed on a biotin-SAM formed on the sensor surface. Then, biotin-BSA was flowed onto the surface (indicated by the first arrowhead), and BSA was trapped on the streptavidin layer through the biotin/streptavidin reaction. Afterward, streptavidin and biotin-BSA solutions were alternately applied to the surface. The refractive index increased in a stepwise manner indicating the formation of a multilayered membrane composed of streptavidin and biotin-BSA. After this procedure was repeated 10 times, the thickness of the multilayered membrane was about 30 nm as indicated by an SPR shift when the refractive index of the BSA and streptavidin layer was assumed to be 1.45.

Modifying the Surface of Islets with the Multilayered Membrane. In order to construct a biotin-BSA/streptavidin multilayer on the membranes of islets, a hydrophobic lipid chain conjugated hydrophilic PEG chain carrying a biotin molecule was applied to the cell suspension. When a biotin-PEG-lipid dispersion is mixed with the islets suspension, it is expected to incorporate into cell membranes spontaneously via hydrophobic interactions between the hydrophobic part of biotin-PEG-lipid and the lipid bilayer of the cell membrane. Hydrophilic PEG chains remain on the outer surface of the cell, as illustrated in Scheme 1.

First, the surface of single CCRF-CEM cells (floating cells, acute lymphoblastic leukemia T-cells) was treated with biotin-PEG-lipid to confirm the occurrence of the reaction. After incubation of the cells in biotin-PEG-lipid dispersion, FITC-labeled streptavidin was applied. Figure 2a shows the microscopic images of cells modified with FITC-labeled streptavidin/biotin-PEG-lipid. Fluorescence from FITC-streptavidin was visible at the periphery of the each cell, indicating that FITC-streptavidin had formed a complex with the biotin-PEG-lipid on the cell membrane. No fluorescence was observed for cells not treated with biotin-PEG-lipid. Thus, the hydrophobic lipid of the biotin-PEG-lipid was anchored in the lipid bilayer of the cell membrane as shown in Scheme 1. Cell viability as assessed by Trypan blue exclusion was 89% after the modification. There was no statistically significant difference in cell viability between cells modified with streptavidin/biotin-PEG-lipid conjugates and cells prior to modification. Therefore, the surface modification was not toxic to the living cells.

Islets were sequentially treated with biotin-PEG-lipid and FITC-streptavidin. Their fluorescent images taken with a confocal laser-scanning microscope are shown in Figure 2b. Clear fluorescence is seen at the periphery of each islet; no fluorescence was observed on islets treated with FITC-streptavidin when the biotin-PEG-lipid treatment was skipped. These observations indicate that biotin groups can be introduced on the surface of islets using biotin-PEG-lipid and that streptavidin can be anchored to the islet surface via biotin without damaging islet morphology. To form a multilayered membrane of streptavidin and biotin-BSA, the biotinylated islet surface was treated sequentially with streptavidin and biotin-BSA; this procedure was repeated several times. Finally, FITC-streptavidin was used for the 21st layer to allow visualization under a confocal laser-scanning microscope. Figure 2c shows fluorescent images of islets encapsulated with 21 layers of biotin-BSA and streptavidin. A strongly fluorescing layer was observed at the periphery of all islets, indicating the presence of multilayered membranes of biotin-BSA and streptavidin.

Immobilization of Bioactive Molecules to the Surface of Encapsulated Islets. Urokinase is a serine protease that activates plasminogen. Activation of plasminogen triggers a proteolysis cascade that participates in thrombolysis. Immobilizing urokinase on the surface of islets is expected to help dissolve small blood clots that may form on islets, thereby suppressing blood-mediated inflammatory reactions. Multilayered membrane-modified islets were further modified with dialdehyde dextran to introduce aldehyde groups on the surface of islets. Urokinase was immobilized on the islets surface through Schiff base formation between the aldehyde groups with primary amines of urokinase. FITC-labeled BSA was reacted with the aldehyde groups in place of urokinase, allowing direct visualization (Figure 2d). Fluorescence from FITC-BSA was observed at the periphery of all islets, indicating the presence of FITC-BSA. On the other hand, no fluorescence was observed on islets without treatment with activated dextran (Supporting Information Figure S2 (a)). Islets after treatment with biotin-BSA and streptavidin were also used to immobilize heparin at the surface. Protamine was immobilized on islets with biotin-BSA as an outermost surface and then anionic heparin was immobilized on the protamine layer through polyion complex formation. Fluorescence from FITC-heparin was observed at the periphery of all islets (Figure 2e), indicating the presence of FITC-heparin on the islets. There was no fluorescence on islets without the protamine layer (Supporting Information Figure S2 (b)). Thus, our technique enables various bioactive molecules to be immobilized on the surface of islets.

A fibrin plate-based assay was performed to assess the function of the urokinase anchored on the islets. Urokinase transforms plasminogen into active plasmin, which can dissolve fibrin. To assess the activity of the membrane-anchored urokinase, we spotted the modified islets on a fibrin gel, and measured the area of dissolved fibrin around the spotted urokinase-islets. Figure 3a shows the fibrin plate at 12 h after urokinase-islets spotting. A large transparent area was observed around the urokinase-islets, indicating urokinase activity. The transparent area was small around naïve islet spots. The small transparent area might be due to small amounts of urokinase secreted by the islet itself (*18*). Islets with anchored urokinase were cultured in Medium 199 supplemented with 10% FBS. Urokinase activity during culture was followed and is shown in Figure 3b. The fibrinolytic activity of urokinase was retained for 3 days, and then decreased to 25% after 7 days in culture. The stability of the surface modifications was also examined by observing the morphology of islets treated with a multilayered membrane (total layer number = 20). Their outermost layer was treated with FITC-streptavidin and fixed with dialdehyde dextran. Figure 4

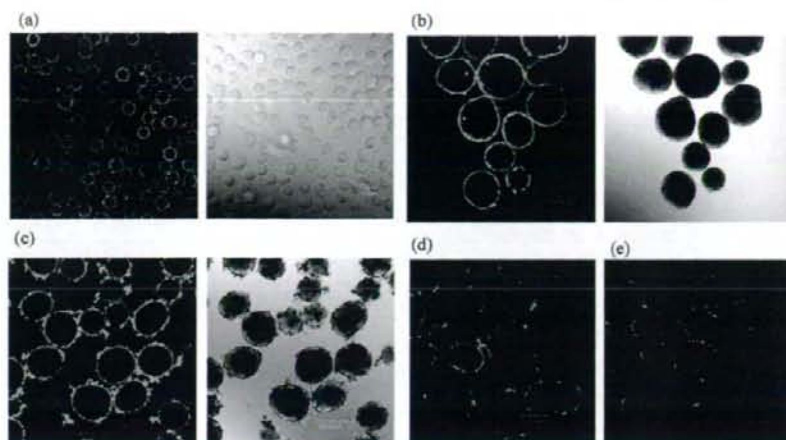


Figure 2. Images of surface-modified cells and islets observed under confocal laser and differential interference microscopy. (a) CCRF-CEM cells were sequentially modified with biotin-PEG-lipid and FITC-labeled streptavidin. (b) Hamster islets were sequentially modified with biotin-PEG-lipid and FITC-labeled streptavidin. (c) Hamster islets modified with biotin-PEG-lipid were sequentially coated with streptavidin and biotin-BSA (20 times); the 21st layer was FITC-streptavidin. (d) Islets covered with a multilayered biotin-BSA/streptavidin membrane were activated with oxidized dextran. FITC-BSA was anchored to the islets through Schiff base formation. (e) Multilayered islets were coated with cationic protamine and FITC-labeled heparin (anionic) was immobilized on the islets through polyion complex formation. Scale bars: 20 μm for (a), and 200 μm for (b), (c), (d), and (e).

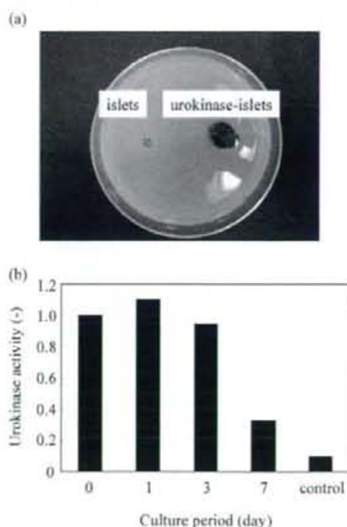


Figure 3. Fibrin plate assay for islet-anchored urokinase. (a) 100 naïve islets and 100 urokinase-modified islets were spotted on the left and right sides of a fibrin plate, respectively, and incubated at 37 °C for 12 h. (b) Bar graph illustrating the change in islet-anchored urokinase activity during culture in medium. Activity is expressed as the area of dissolved fibrin around urokinase-islet spots normalized to the area at day zero (see Experimental Section).

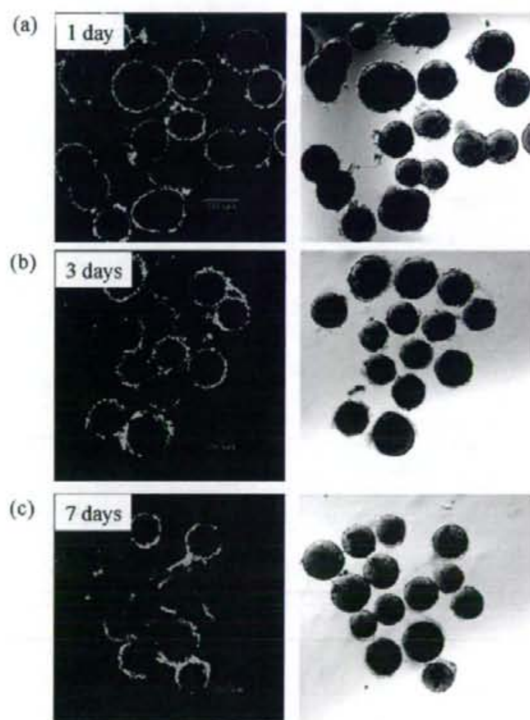


Figure 4. Changes in multilayered membranes on islets after (a) 1, (b) 3, and (c) 7 days in culture. Islets had been coated with a multilayered biotin-BSA/streptavidin membrane on biotin-PEG-lipid modified surface, followed by reaction with activated dextran and culture in Medium 199.

shows the microphotographs of islets at 1, 3, and 7 days of culture. The membrane gradually deteriorated over time, although islet morphology was well-maintained. Thus, urokinase could have detached from the islets, which could account for the decrease in urokinase activity.

Glucose-Stimulated Insulin Release by Surface-Modified Islets. A glucose stimulation test was performed in order to examine the ability of the modified islets to control insulin release in response to glucose level changes; the results are summarized in Figure 5. When the glucose concentration was increased from 0.1 g/dL to 0.3 g/dL, islets in all groups increased insulin release from basal levels. Insulin release returned to the basal level when islets were re-exposed to 0.1 g/dL, indicating

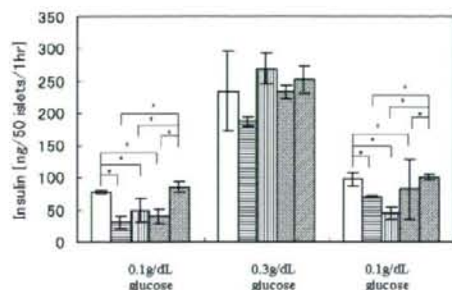


Figure 5. Glucose stimulation test. Bars from left indicate (1) islets without surface modification, (2) islets modified with a multilayered biotin-BSA/streptavidin membrane, (3) periodate-activated dextran-treated islets, (4) urokinase-anchored islets at day zero, and (5) urokinase-anchored islets after 7 days of culture in Medium 199 at 37 °C. The amounts of insulin secreted from modified islets in response to glucose concentration changes (0.1, 0.3, and 0.1 g/dL) were determined by ELISA. ($n = 3$). * indicates a statistically significance difference ($p < 0.01$).

that islets after surface modification normally response to the glucose changes. At low glucose level, the insulin secretion rates in groups of islets after surface modification are slower than that of control group, whereas it was the same level among all groups at high glucose level. However, the all insulin secretion rates were recovered to the basal level after seven days culture. These results indicate that the surface modifications and urokinase anchoring did not influence the islets' ability to regulate and release insulin.

DISCUSSION

Recently, Nilsson et al. reported that the graft survival of porcine islets could be improved by modifying the surface of islets with heparin (*12*), and Chaikof et al. reported immobilizing thrombomodulin to the surface of islets (*19*). In their approaches, biotin molecules were conjugated to amino groups of islets surface proteins in order to cover the surface with avidin for immobilizing heparin, and it is necessary to covalently conjugate phosphine molecules to surface protein amino groups to anchor thrombomodulin. Although they reported some improvements in the graft survival by their modification methods, their method is not sufficient enough to solve all of the problems related with the graft loss in the islet transplantation. It is meaningful to increase options to modify islet surface.

In this study, we develop a versatile method for anchoring bioactive substances onto the surface of islets. First, biotin-PEG-lipid was anchored to the lipid bilayer of the cell membrane through hydrophobic interactions. This modification had no influence on cell viability. The biotin molecule on the cell surface was used to add multilayered membranes of streptavidin and biotin-BSA. Then, a fibrinolytic enzyme, urokinase or anticoagulant heparin, was immobilized to the outermost surface to prevent the instant blood-mediated inflammatory reaction. Our methods can much more easily immobilize enzymes and polysaccharide on islet surfaces. The surface modifications did not influence the glucose-responsive insulin-secreting ability of islets, and anchored urokinase retained its fibrinolytic properties at least for 7 days. Therefore, thrombosis that form immediately after transplantation would be prevented from using these modified islets for transplantation. Immobilizing urokinase and heparin on the surface of islets is an attractive method because it would prevent systemic administration of anticoagulants, which is associated with an increased risk of bleeding after intraportal transplantation.

As shown Figure 2c, a strong fluorescence layer was observed at the periphery of all islets. Multilayered membranes of biotin-BSA and streptavidin seem to be formed on islets from the microphotographs and SPR monitoring shown in Supporting Information Figure S1. Although the surface of islets treated with a single layer was smooth, the surface became rough and some debris-like material was found on the surface of islets coated with the 21 layers. The internal tension in the coated membranes is expected to increase with a number of the layer-by-layer sequences. The layer formed was peeled off when the internal tension became larger than the anchoring strength of the PEG-lipid into the lipid bilayer. Bovine serum albumin is a globular protein and is expected not to form a mechanical strong film. We will examine synthetic linear polymers, such as poly(vinyl alcohol) and cellulose derivatives, which have been used to prepare tough membranes in industrial applications.

The number of amino groups in urokinase (25 lysine residues in a molecule) is less than that of BSA (59 lysine residue in a molecule) and the reaction used to immobilize proteins was Schiff base formation which is considered to be a reversible reaction. Although urokinase seems to be easily released from the islet surface, the fibrinolytic activity of urokinase immobilized on islets retained 25% of its initial activity after 7 days in culture. Activated dextran carries a number of aldehyde groups in a molecule. The interaction between the activated dextran and urokinase becomes stronger and more stable due to multibond formation between these molecules, even though each Schiff base is unstable by itself. Our technique using activated dextrane enables various bioactive proteins to be immobilized on the surface of islets.

Islets microencapsulated within an alginate-poly-(L-lysine) membrane and an agarose hydrogel membrane have been investigated for use as a bioartificial pancreas (*20–26*). Many groups have reported that a long-term normoglycemia in a diabetic small animal, such as a mouse or a rat, can be realized by transplanting microencapsulated islets into its peritoneal cavity. However, in clinical settings, about 10 mL of islet suspension should be injected through a catheter into the portal vein in liver. The diameter of microencapsulated islets was several times larger than that of islets, which could result in plugged vessels if infused into the portal vein. As shown in Figure 1 for SPR examinations, the thickness of the membrane increases stepwise using the streptavidin/biotin-BSA multilayer method. We examined the possibility of enclosing islets within a thin membrane using this layer-by-layer method. After forming 21 layers, the multilayer thickness reached tens of nanometers, as evaluated by SPR method and microscopy (Figures 1 and 2). The thickness was enough to the cover islet surface, but the islets' volume increase was small. Our method holds promise as a means to enclose islets without increasing volume. Immunoisolative efficacy of the layer-by-layer membrane is carefully being examined in islet transplantation studies; the results will be reported.

We examined membrane stability and found that the membrane gradually deteriorated over time (Figure 4). Either the mechanical strength of the streptavidin and biotin-BSA membrane is not strong enough for long-term encapsulation or enzymes secreted from the cells degraded the membranes. In addition, it would be necessary to carefully examine the effect of urokinase and heparin immobilized to the surface of islets *in vivo*. Future work will focus on maintaining the integrity of the multilayer membrane for longer periods and graft survivals will be carefully examined.

In conclusion, thin-layered membranes were formed on the surface of islets via biotin-PEG-lipids and the biotin/streptavidin system without increasing cell volume. Further, urokinase was anchored to the membrane, and its high fibrinolytic ability was maintained for seven days in culture. This novel method for

constructing a bioartificial pancreas provides a promising means for improving graft survival following intraportal transplantation.

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Supporting Information Available: Additional information as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Cryopreserved Agarose-Encapsulated Islets As Bioartificial Pancreas: A Feasibility Study

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Background. A bioartificial pancreas in which islets of Langerhans (islets) are encapsulated within a semipermeable membrane, such as agarose, has been proposed for treating type I diabetic mellitus. However, the long-term storage for providing a convenient and easily accessible supply still remains an issue. We investigated cryopreservation as a potential method of long-term storage for agarose-encapsulated islets (Mic-islets).

Methods. The morphology, insulin secretion, and histochemical staining of cryopreserved Mic-islets were analyzed. Streptozotocin-induced diabetic mice were transplanted intraperitoneally with 1000, 2000, and 3000 Mic-islets after cryopreservation in KYO-1 vitrification solution. Blood glucose levels were measured and immunohistochemical analyses were performed at 41 days posttransplantation.

Results. Transplanted cryopreserved Mic-islets restored normoglycemia in diabetic mice. The mean (±SD) normoglycemic periods were 32.0±13.2 days and 46.3±13.3 days for recipients of 1000 (n=5) and 2000 (n=4) cryopreserved Mic-islets, respectively, whereas the mean normoglycemic period was 53.2±16.7 days for recipients of 1000 noncryopreserved Mic-islets (n=7). These data indicate that cryopreserved Mic-islets transplanted as a bioartificial pancreas successfully controlled blood glucose levels for extended periods.

Conclusion. Cryopreserved agarose-encapsulated islets could successfully control the blood glucose level for a long period as a bioartificial pancreas.

Keywords: Bioartificial pancreas, Cryopreservation, Vitrification, Islets, Transplantation, Agarose.

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Islets of Langerhans (islets) transplantation has been gradually accepted as an effective treatment method for type 1 diabetes mellitus. The Edmonton protocol improves restoration of insulin production and glycemic stability after intraportal islets transplantation (*1*). Microcapsule-type bioartificial pancreas have been evaluated by some groups (*2–5*), and our group originally developed a bioartificial pancreas in which islets are microencapsulated within agarose hydrogel as a candidate for transplantation (*6–9*). We reported that agarose-encapsulated islets provide long-term restoration of normoglycemia in diabetic model animals after allogeneic or xenogeneic islets transplantation. Despite a number of successful reports regarding this procedure, several issues need to be resolved before bioartificial pancreas becomes an accepted medical device. One of the requirements is a banking system that allows the long-term storage of bioartificial pancreas containing large quantities of living islets, and one possible route is cryopreservation.

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Cryopreservation of living cells has been performed using dimethyl sulfoxide (DMSO) as a cryoprotectant. Although these conventional cryoprotectants and the use of various cooling and warming rates have allowed the successful cryopreservation of islets (*10–14*), it is difficult to preserve the integrity of the capsule membrane. To avoid inter- and intracellular ice formation and to preserve material integrity, vitrification might be more advantageous for bioartificial pancreas storage. Vitrification is the solidification of a liquid that contains a high concentration of cryoprotectant into a glass state without crystallization. Recently, we examined the cryopreservation of agarose-encapsulated islets (Mic-islet) using a newly developed vitrification solution called KYO-1 and reported their morphology and insulin secretion functions before and after cryopreservation (*15*). With KYO-1, the Mic-islets were viable and restored insulin secretion ability after vitrification and warming; in addition, the mechanical and physical-chemical properties of the hydrogel were well preserved during culture "in vitro."

In this article, we evaluated "in vivo" functions of cryopreserved Mic-islets as a bioartificial pancreas. Cryopreserved Mic-islets were transplanted into the intraperitoneal cavities of streptozotocin-induced diabetic mice to evaluate the ability of the agarose beads to control blood glucose metabolism and to evaluate their immunoisolation properties in vivo as compared with noncryopreserved Mic-islets. We used a xenogeneic transplantation model (*16*), in which agarose bead-encapsulated hamster islets were transplanted to the diabetic mice. In this xenogeneic transplantation model, the long-term restoration of normoglycemia could be realized in transplantation of agarose bead-encapsulated hamster islets by the administration of low dose of immunosuppressant 15-deoxyspergualin. Therefore, we could discern how many cells in the islets survived the cryopreservation process by insulin secretion tests and by the period of normoglycemia induced

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by transplantation of various numbers of cryopreserved Mic-islets in comparison with normal Mic-islets using this xenotransplantation model. In addition, we evaluated the immunoisolation efficacy and the stability of the cryopreserved agarose beads in vivo.

MATERIALS AND METHODS

Microencapsulation of Islets in Agarose Hydrogel

Syrian hamsters were used as donors of pancreatic tissue (7–8 weeks of age, female, Japan SLC, Inc., Shizuoka, Japan). Islets were isolated by the collagenase digestion method (*17*) and cultured for 6 days in culture medium (Medium 199 [Invitrogen Co., Carlsbad, CA]) with 10% fetal bovine serum (FBS), 8.8 mM HEPES buffer, 100 U/mL penicillin, 100 μg/mL streptomycin, and 8.8 U/mL heparin. The islets were then encapsulated in agarose hydrogel according to previously described protocols (*6, 7*). Briefly, a 5% agarose solution (Taiyo Agarose, AG LT-600, Shimizu shokuhin KK, Shimizu, Japan) in serum-free minimum essential medium (3 mL, minimum essential medium, Invitrogen) was autoclaved in a 50-mL glass centrifuge tube, then maintained at 40°C for 8 min. Next, 700 to 1000 islets were mixed with the agarose solution and 15 mL of 40°C liquid paraffin (Merck Co & Inc, Germany) were added. The mixture was suspended by manual shaking to form agarose droplets then immersed in an ice bath for 5 min with gentle agitation to provoke gelling of the agarose droplets. Afterward, 15 mL of cooled Hanks' balanced salt solution (Invitrogen) was added to the glass tube and the suspension was centrifuged at 2000 rpm for 5 min at 4°C. The supernatant liquid paraffin phase was removed. This procedure was repeated three times; then, microbeads containing islets were handpicked and cultured in medium under 5% CO_2 at 37°C for 1 day before cryopreservation.

Cryopreservation of Mic-Islets in Vitrification Solution

Mic-islets were cryopreserved as previously described (*15*). Briefly, 1000 agarose-encapsulated Mic-islets were placed in a cryotube and suspended in 100 μL of Euro-Collins solution (34.95 g/L dextrose, 7.3 g/L K_2HPO_4, 2.04 g/L KH_2PO_4, 1.12 g/L KCl, and 0.84 g/L $NaHCO_3$). KYO-1 (*15*), which consists of 5.38 M ethylene glycol, 2 M DMSO, 0.1 M PEG 1000, and 0.00175 M PVP K10 (MW 10,000) in Euro-Collins, was used as a vitrification solution. When KYO-1 was used, the concentration was previously concentrated by 1.1. Cryopreservation was a multistep process: First, 10 μL of cooled KYO-1 was added and the Mic-islets were incubated for 10 min at room temperature (RT) resulting in a final concentration of 10.0% KYO-1. Next, 5 μL of cooled KYO-1 was added and Mic-islets were incubated for 15 min at RT, resulting in a 14.3% concentration of KYO-1. Then 15 μL of cooled KYO-1 was added and Mic-islets were incubated for 15 min at 0°C, resulting in a 25.4% concentration of KYO-1; the mixture was resuspended every 5 min. Finally, 980 μL of KYO-1 was added and Mic-islets were incubated for 5 min at 0°C, resulting in a 100% concentration of KYO-1. After that cryotubes were placed in a freezing machine (Planar Kryo 360-1.7, Planer Products Ltd., U.K.) in which the cooling and warming rates can be controlled. The cryotubes were cooled rapidly (−43°C/min) to −150°C, followed by a slow cooling (−3°C/min) to −185°C. To monitor exo- and endothermal phenomena and to monitor the cooling rate, thermometers were inserted both in a separate dummy sample containing the KYO-1 solution and in the chamber itself. Finally, cryotubes were submerged into liquid nitrogen and stored until use.

Warming of Cryopreserved Mic-Islets

Cryopreserved Mic-islets were warmed in two stages. First, cryotubes were warmed to −100°C (30°C/min) in the freezing machine. Then, they were immersed in 30% DMSO in water at RT (225°C/min) for rapid warming (*18*). Afterward, a suspension of Mic-islets was transferred to a conical tube containing 10 mL of Euro-Collins solution and centrifuged at 1200 rpm for 3 min. The supernatant was discarded and the Mic-islets were resuspended in 0.75 M sucrose solution (1 mL in RPMI-1640 supplemented with 10% FBS) and incubated at 0°C for 30 min to remove intracellular vitrification solution; the mixture was resuspended every 5 min by gentle shaking. Then, ice-cold culture medium (1 mL, RPMI-1640 supplemented with 10% FBS) was added each 5 min for 20 min. The final suspension was centrifuged at 1200 rpm for 3 min and resuspended in medium for culture at 37°C.

Evaluation of Cryopreserved Mic-Islets "in vitro"

Static insulin secretion tests were performed on noncryopreserved and cryopreserved Mic-islets 1 day after warming and after 1 week in culture to evaluate their insulin secreting ability in response to changes in the glucose concentration. Mic-islets were exposed to 0.1 g/dL, then 0.3 g/dL, and finally 0.1 g/dL glucose in Krebs-Ringer solution for 1 hr each at 37°C. The solutions were collected after the 1-hr incubation for each glucose concentration. Insulin concentrations in the solutions were determined by enzyme-linked immunosorbent assay (Shibayagi Co. Ltd., Gunma, Japan).

Evaluation of Cryopreserved Mic-Islets "in vivo"

Balb/c mice were used as recipients of cryopreserved Mic-islets. Diabetes was induced in Balb/c mice (male, 6-weeks of age; Japan SLC, Inc.) by intraperitoneal injection of streptozotocin (230 mg/kg body weight; Nacalai tesque INC, Japan) 10 days before transplantation. An animal was considered diabetic when its plasma glucose level exceeded 400 mg/dL in two consecutive measurements. The immunoisolation efficacy of agarose encapsulation was not sufficient to protect the hamster islets between hamster from the immunologic systems of the mice; therefore, the immunosuppressant 15-deoxysperguarin (Nippon Kayaku, Tokyo, Japan) was injected daily intraperitoneally (5.0 mg/kg/day) beginning 3 days before transplantation (*7*).

The first group of mice received 1000 Mic-islets without cryopreservation (control group, n=7). The second group received 1000 cryopreserved Mic-islets (n=5). The third group received 2000 cryopreserved Mic-islets (n=4). For intraperitoneal glucose tests and histochemical analyses, 3000 cryopreserved Mic-islets were transplantation into two

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