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２１年度　総括研究報告書

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（総括）研究報告書

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

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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（膵島）の移植が注目されている。エドモントンプロトコールによ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など、様々な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）と生理活性物質（ウロキナーゼ）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 脂質とウロキナーゼ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。膵島の表面を PEG 修飾あるいはウロキナーゼで修飾することで、移植直後に生じ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、臨床応用可能な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すものである。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)カプセル化に伴う体積が著しく増加すること、(2)移植後に副作用(血栓症や炎症反応)が起き、細胞障害へと繋がることである。これらの問題点は、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。また、高分子膜に線用系活性酵素であるウロキナーゼを担持させ、抗血栓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)を利用したものである。生着率を高める目的でウロキナーゼ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）を利用してウロキナーゼを固定した。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（BALB/c マウス）へ、PEG 脂質を用いて膵島表面（BALB/c マウス由来）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。また、さらにウロキナーゼを固定化した膵島を移植した。未処理の膵島群をコントロール群として、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。

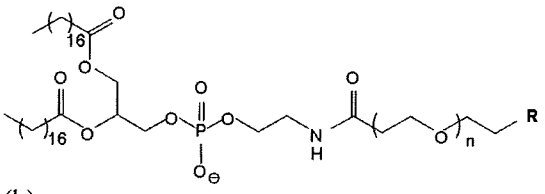
（倫理面への配慮）

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。

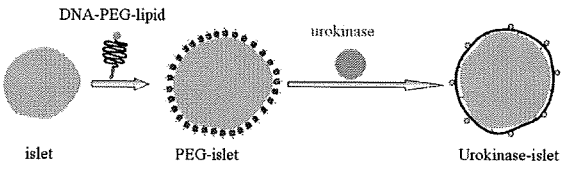
C. 研究結果

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。この表面修飾法は、細胞障害を引き起こさず、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。図1(a), (b)に示したように、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修飾や線用系活性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。

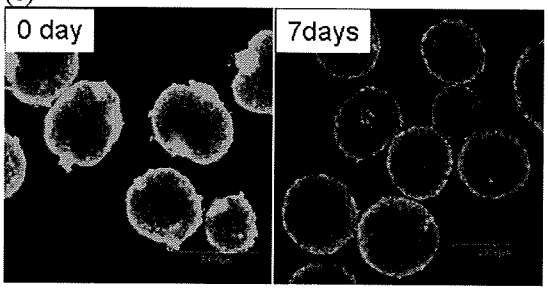
(a)



(b)



(c)



(d)

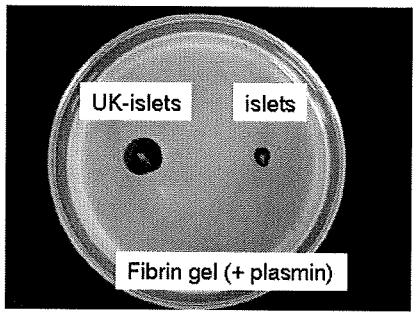


図 1. PEG 脂質による膵島の表面修飾とウロキナーゼ固定化膵島の評価 (a) PEG 脂質の化学構造式, (b)表面修飾の方法(c) FITC 標識 PEG 脂質で表面修飾したマウス膵島の経時観察（培養液中）, (d) ウロキナーゼ固定化膵島のフィブリン溶解活性の評価

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(c)）。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。ただ、時間の経過とともに、膵島表面から PEG 脂質の脱離がみられた（図 1©）。このことは、移植直後で起きる炎症反応のみを抑制できることを示唆するものである。

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（Data not shown)。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あるいはウロキナーゼを固定化した膵島でも、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。

また、ウロキナーゼを固定化した膵島の機能評価を行うために、フィブリンゲルの溶解活性を利用して評価を行った（図 1(d)）ウロキナーゼ固定化膵島の周辺のフィブリンゲルが溶解していることから、膵島表面に固定化しているウロキナーゼの活性が保持されていることが分かる。このように、PEG 脂質を利用してウロキナーゼを固定化した膵島を移植実験に使用した。

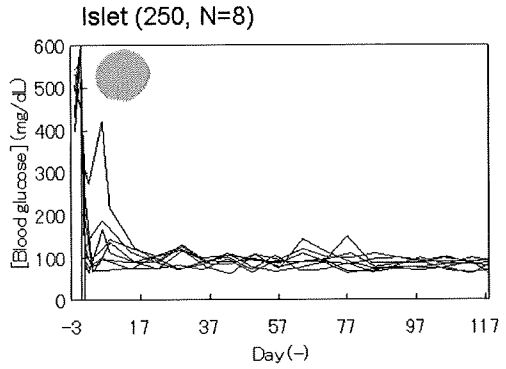
糖尿病マウスの肝臓内へ門脈を通して、BALB/cマウス由来の膵島を移植した。この移植モデルは、免疫拒絶がおきない同種同系移植モデルとなっている。移植後におきる炎症反応による影響を調べるために、本モデルを選択した。本実験では、PEG脂質

あるいはウロキナーゼ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いる。移植数は、未処理の膵島を125個と250個、PEG脂質で修飾した膵島あるいはウロキナーゼを固定化した膵島を125個移植した。それぞれの血糖値変化を図2にそれぞれ示す。未処理の膵島を移植した場合では、250個移植した場合（図2(a)）では、全てのマウスの血糖値が正常値を示しており、移植した膵島が生着し機能していることを示している。ただ、移植数を125個まで減少させると（図2(b)）、半数の糖尿病マウスでは、血糖値が正常化されたものの、残りの半数のマウスでは、血糖値が正常化されず、糖尿病のままであった。このことは、移植した膵島が障害を受けて崩壊し、残存している膵島では血糖値を正常化することができなかったこと示している。

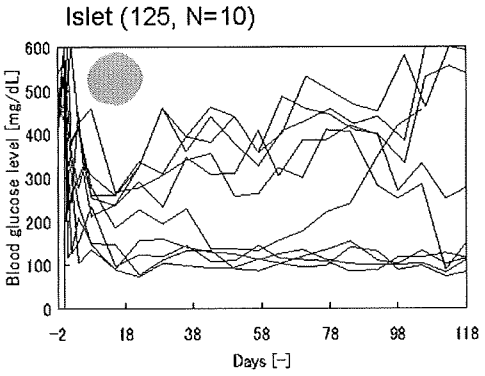
他方、PEG-膵島を125個移植した場合では（図2(c)）、移植後しばらくは、血糖値が不安定であるものの、全てのマウスにおいて、正常血糖値を示した。また、ウロキナーゼを固定化した膵島を125個移植した場合においても(図2(d))、全てのマウスにおいて、正常血糖値を示した。このことは、表面修飾により、移植後の細胞障害が軽減されて、残存する膵島で血糖値を正常化することが可能であることを示す結果である。

以上のことから、PEG修飾あるいはウロキナーゼの固定化により、移植直後の炎症反応が関与する細胞障害が抑制でき、生着率を向上できることが分かった。

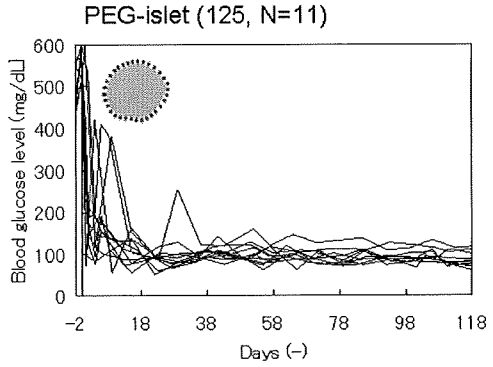
(a)



(b)



(c)



(d)

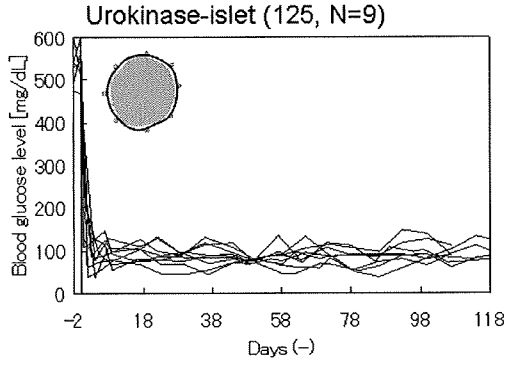
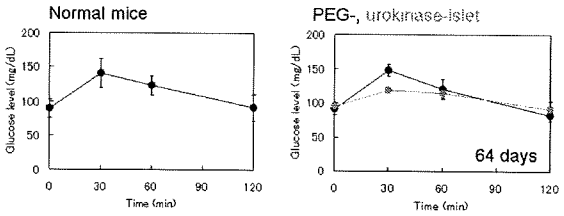


図 2. 膵島移植後の糖尿病マウスの血糖値変化 (a)未処理の膵島（250 islets），(b) 未処理の膵島（125 islets），(c) PEG 脂質で表面を修飾した膵島（125 islets），(d) ウロキナーゼを固定化した膵島（125 islets）

PEG 修飾した膵島あるいはウロキナーゼ固定化膵島を、門脈を通して肝臓内へ移植した後、64 日後にグルコース負荷試験（IPGTT）を行った。その結果を図 3(a)に示す。また、その後、犠牲死させて、肝臓の

組織学検査（HE 染色、インスリン染色）を行った（図 3(b)）。図 3(a)で示すように、正常マウスの応答と同じように、膵島移植群でも血糖値の応答が見られ、移植した膵島が 64 日後でも正常に機能して生着していることを示している。このことからも、表面修飾した材料が膵島の機能へ与える影響はほとんど無いものと考えられる。また、HE 染色像あるいはインスリン染色像からも、移植した肝臓の血管外へ生着していることが分かった。

(a)



(b)

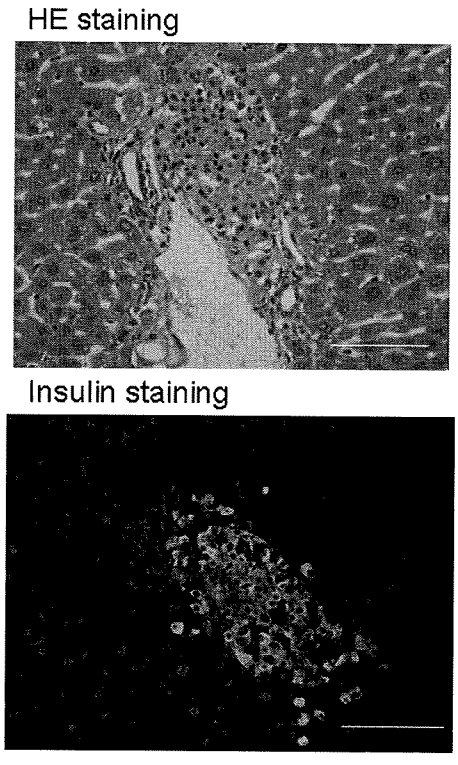


図 3. マウスのグルコース応答試験（IPGTT）。対象は、正常マウス、PEG 脂質修飾膵島あるいはウロキナーゼ固定化膵島の移植したマウス　(b) 膵島移植後（64 日後）の糖尿病マウスの肝臓の HE 染色像とインスリン染色像

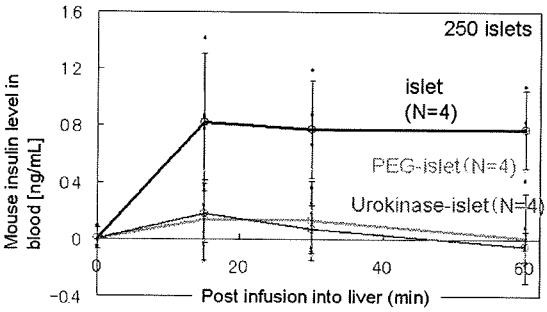


図 4. 膵島移植後のマウス血中インスリン濃度変化. ○: 未処理の膵島移植群(250 islets), ○: PEG 脂質で表面修飾した膵島移植群(250 islets), ○: ウロキナーゼ固定化膵島移植群(250 islets) .

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-膵島あるいはウロキナーゼ固定化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修飾あるいはウロキナーゼ固定化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、表面修飾により、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脂質と生理活性物質であるウロキナーゼ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。さらに、線用系活性酵素であるウロキナーゼを固定化することで、機能を付与することができ、血液適合性を向上することができる。

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。これらの報告結果と本実験結果も合致するため、PEG修飾の効果により膵島の生着率が向上したものと考えられた。

本実験では、PEG脂質あるいはウロキナーゼにて表面修飾した膵島を糖尿病マウスへ移植した。グラフトが消失するまでの生着期間について、未処理の膵島郡と比較している。PEG修飾した膵島あるいはウロキナーゼ固定化膵島を移植した場合では、グラフトの生着率が向上することが分かった。本実験からは、ウロキナーゼの効果を明確に示すことができないものの、移植後の血糖値は比較的PEG修飾群よりも安定していることから、血栓の溶解を引き起こすことがこのような結果につながったものと考えている。

E. 結論

膵島表面にPEG修飾あるいはウロキナーゼを固定化することで、移植直後に生じる生体適合性を向上でき、膵島への細胞障害が著しく軽減できた。このことは、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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特になし

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Yuji Teramura Hiroo Iwata	Bioartificial pancreas - Microencapsulation and conformal coating of islet of Langerhans -	*Advanced Drug Delivery Reviews*		印刷中	
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Surface Modification of Islets With PEG-Lipid for Improvement of Graft Survival in Intraportal Transplantation

Yuji Teramura[1,2,4] *and Hiroo Iwata*[3]

Background. Transplantation of islets of Langerhans (islets) is a promising technique for treating insulin-dependent diabetes mellitus (type I). One unsolved issue is the early graft loss due to inflammatory reactions triggered by blood coagulation and complement activation that occurs immediately after transplantation into the liver through the portal vein. Several proposed approaches for improvement of the graft survival include heparin coating and covalent poly-(ethylene glycol) (PEG) conjugation. We previously have studied the improvement of graft survival by modification of islet surfaces using amphiphilic PEG-conjugated phospholipid and bioactive molecules. Here, we analyzed the effect of PEG-modification on the improvement of graft survival immediately after intraportal transplantation into streptozotocin-induced diabetic mice.

Methods. The surface of hamster islets was modified with PEG-lipid. PEG-lipid modified islets (PEG-islets) were transplanted into the liver through the portal vein of streptozotocin-induced diabetic mice. We measured the graft survival periods and blood insulin levels immediately after intraportal transplantation to determine the cell damage to islets. Histocytochemical analyses of liver were also performed postintraportal transplantation.

Results. The graft survival of PEG-islets was significantly prolonged compared with bare islets in livers of diabetic mice. Reduction of blood insulin level within 60 min after transplantation of PEG-islets suggests that the cell damage observed immediately after transplantation could be suppressed by surface modification with PEG in comparison with bare islets.

Conclusion. Our approach for the improvement of graft survival will be useful in the clinical setting.

Keywords: Bioartificial pancreas, Islets, Poly(ethylene glycol)-lipid, Intraportal transplantation, Graft survival.

(*Transplantation* 2009;88: 624–630)

Although transplantation of islets of Langerhans (islets) is still an experimental procedure, this approach has been proven to be a safe and effective method for treating patients with insulin-dependent diabetes mellitus (type I). Notably, the success achieved with the Edmonton protocol has established clinical islet transplantation as an alternative to pancreas transplantation (*1*, *2*). However, some aspects of islet transplantation remain to be improved, including increase of islet isolation efficiency, improvement in islet preservation, increase in the efficacy of immunosuppressive drug dosage protocols, and reduction of islet loss in the early phase after transplantation. Among these issues, graft loss poses the most serious limitations for current islet transplantation protocols. Transplantation of islets from a single donor pancreas is not sufficient to achieve normoglycemia in a patient (*2*), suggesting that many islets are lost soon after intraportal transplantation.

Innate immune reactions are involved in the destruction of islets exposed to fresh blood in the portal vein (*3–7*). Blood coagulation and complement systems are activated on exposure of islets to fresh blood components; chemotactic factors, tissue factor, chemokines, and other inflammatory mediators are released during this early phase, which induce instant blood-mediated inflammatory reactions resulting in graft loss (*5*). Some approaches to regulate early coagulation and blood-mediated inflammatory reactions, such as administration of Melagatran (*6*) and dextran sulfate (*7*), have been

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Yuji Teramura: research design, the writing of the manuscript, the performance of the research, and data analysis, and Hiroo Iwata: research design and the writing of the manuscript.

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studied, and suppression of these reactions has improved transplantation success by reducing islet loss in animal models. However, it is difficult to apply these methods to the clinical situation because systemic administration is associated with a risk of bleeding. Several methods have been reported for improvement of graft survival in the liver, such as coating of islet surface with polymers and bioactive molecules (*8*, *9*). Contreras et al. (*8*) reported the improvement of graft survival in the liver by covalent conjugation of poly(ethylene glycol) (PEG) to the islet surface and transfection of an apoptosis-suppressing gene. Recently, Nilsson and coworkers (*9*) proposed a new method for preventing instant blood-mediated inflammatory reactions by coating islets with heparin to inhibit surface thrombosis formation after transplantation.

We previously studied the effect of surface modification of living cells and islets using amphiphilic polymers such as PEG-conjugated phospholipid and poly(vinyl alcohol) with alkyl side chains on the improvement of graft survival during cell transplantation (*10–14*). The results from our methods are promising because PEG-lipid derivatives can be immobilized to the cell surface by hydrophobic interactions between the lipid bilayer membranes. Furthermore, a thin PEG layer can form on the islet surface without cytotoxicity and volume increase.

This article evaluates the efficacy of PEG-modification on the improvement of graft survival immediately after transplantation in the liver through the portal vein of streptozotocin (STZ)-induced diabetic mice. We predicted that surface modification with PEG could suppress the coagulation reactions and inflammatory reactions that mediate islet loss during the early phase of intraportal transplantation. We studied the graft survival periods, performed histochemical analysis, and measured blood insulin levels immediately after intraportal transplantation to determine the cell damage to islets.

MATERIALS AND METHODS

Materials

α-*N*-Hydroxysuccinimidyl-ω-maleimidyl PEG (M_w 5000) was purchased from Nektar Therapeutics (San Carlos, CA). We purchased 1,2-dipalmitoyl-*sn*-glycerol-3-phosphatidylethanolamine from NOF Corporation (Tokyo, Japan). Dichloromethane, chloroform, *N*,*N*′-dimethylformamide, diethyl ether, STZ, and *tri*-sodium citrate dehydrate were obtained from Nacalai Tesuque (Kyoto, Japan). ICN Biomedicals, Inc. (Aurora, OH) supplied cystein. Fluorescein isothiocyanate (FITC) and Hoechst 33342 were purchased from Dojindo Laboratories (Kumamoto, Japan). Alexa 488-labeled goat anti-guinea pig IgG, minimum essential medium (MEM), HEPES buffer solution, and Medium 199 were purchased from Invitrogen, Co. (Carlsbad, CA). Fetal bovine serum was obtained from BioWest (Miami, FL), and phosphate-buffered saline (PBS) from Nissui Pharmaceutical, Co., Ltd. (Tokyo, Japan). Enzyme-linked immunosorbent assay (ELISA) kits for the insulin assay were from Shibayagi, Co., Ltd. (Gunma, Japan). Tissue-Tek was purchased from Sakura Finetechnical, Co., Ltd. (Tokyo, Japan). Goat normal serum and polyclonal guinea pig anti-insulin were obtained from Dako (Glostrup, Denmark). We purchased 10% formalin solution and Triton X-100 from Wako Pure Chemical (Osaka, Japan). Isoflurene was purchased from Abbott Japan, Co., Ltd. (Japan).

Synthesis of PEG-Conjugated Phospholipid (PEG-Lipid)

Mal-PEG-lipid was synthesized by first dissolving α-*N*-hydroxysuccinimidyl-ω-maleimidyl PEG (180 mg) and 1,2-dipalmitoyl-*sn*-glycerol-3-phosphatidylethanolamine (20 mg) in chloroform and stirring for 24 hr at room temperature (RT). 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, δ 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).

In this experiment, the maleimide group was previously deactivated by the addition of cystein. FITC-labeled PEG-lipid (*12*), which has a similar structure and activity as PEG-lipid, was visualized in islets by confocal laser scanning microscopy (FLUOVIEW FV500, Olympus, Tokyo, Japan).

Preparation of PEG-Lipid-Modified Islets

Islets were isolated from pancreases of Syrian hamsters (7–8 weeks, females, Japan SLC, Inc., Shizuoka, Japan) by collagenase digestion (*15*). The islets were cultured for 4 days after isolation to remove or sediment cells that were damaged during the isolation procedure; cells were cultured in Medium 199 with 10% fetal bovine serum, 8.8 mM HEPES buffer, 100 units/mL penicillin, 100 μg/mL streptomycin, and 8.8 U/mL heparin. A PEG-lipid solution (500 μg/mL in 100 μL of MEM) was added to islets suspended in serum-free MEM, and the mixture was incubated for 1 hr at RT. After washing with serum-free MEM, PEG-lipid-modified islets were transplanted into the livers of diabetic mice.

Static Insulin Secretion Test by Glucose Stimulation

Static insulin secretion tests were performed using 100 PEG-islets to evaluate their ability to secrete insulin in response to changes in glucose concentration. Bare islets were used as a control. Islets were exposed to 0.1 g/dL, then 0.3 g/dL, and finally 0.1 g/dL of glucose in Krebs-Ringer buffer (KRB)solution for 1 hr each at 37°C. The solutions were collected after the 1-hr incubation in each glucose concentration, and insulin concentrations were determined in each solution by ELISA.

Transplantation of PEG-Islets Into Livers of STZ-Induced Diabetic Mice

Balb/c mice were used as recipients of islets and PEG-islets. Diabetes was induced in Balb/c mice (6 weeks, males, Japan SLC, Inc.) by intraperitoneal injection of STZ (230 mg/kg body weight in citrate buffer, pH 4.5) 10 days before transplantation. An animal was considered diabetic when its plasma glucose level exceeded 400 mg/dL in two consecutive measurements.

PEG-islets or control islets (500 or 1000 islets) were transplanted into the liver through the portal vein of STZ-induced diabetic mice, which were anesthetized during surgery by mask inhalation of isoflurane using a specialized instrument (400 Anesthesia Unit; Univentor, Malta); the isoflurane concentration was 4.5% to 5.0% induction and 2.0 for maintenance with airflow rate of 200 mL/min. Diabetic mice were treated with 500 or 1000 control islets or PEG-

islets. Mice were housed in cages with free access to food and water. Nonfasting plasma glucose levels were measured using a glucose sensor (DIAmeter-α glucocard; Arkray, Kyoto, Japan) between 11 A.M. and 1 P.M. before and after transplantation. Blood samples were taken from the subclavian vein. Graft failure was defined as two consecutive plasma glucose level determinations more than or equal to 200 mg/dL. All animal experiments were approved by the Kyoto University Animal Care Committee.

Histochemical Analysis

Mice were killed after transplantation of islets or PEG-islets into the liver through portal vein of diabetic mice for 1 hr or 1 day. Livers were removed, washed with saline, immersed in 10% formalin solution, and incubated for 2 days at RT. The formalin solution was removed, and the livers were sequentially maintained in 3% and 10% sucrose in PBS for 2 days, followed by incubation in 20% sucrose in PBS for 2 days at RT. Livers were then embedded in Tissue-Tek for freezing. The frozen specimens were sliced (6-μm thick) by a cryostat (CM 3050S IV, Leica, Solms, Germany), and the sections were permeabilized with 0.2% Triton X-100 in PBS at RT for 15 min. The samples were then treated with 10% normal goat serum in PBS for 1 hr to block nonspecific binding of antibodies. Sections were treated with 1% polyclonal guinea pig anti-insulin in PBS containing 3% goat normal serum for 3.5 hr at RT, followed by washing with PBS. The samples were then incubated with fluorescent labeled secondary antibody, 0.2% Alexa 488 Goat anti-guinea pig IgG in PBS containing 3% goat normal serum at RT for 1.5 hr. Cell nuclei were counterstained with Hoechst 33342. The stained sections were analyzed using fluorescence microscopy (BX51, Olympus Optical, Co., Ltd., Tokyo, Japan). The sliced sections were also stained with hematoxylin-eosin using a conventional staining method.

Determination of Insulin Levels in Blood After Intraportal Transplantation of PEG-Islets

After transplantation of control or PEG-islets (1000 islets) into the liver of diabetic mice through the portal vein, blood was taken from the subclavian vein at 15, 30, and 60 min and centrifuged to obtain plasma. Hamster insulin in the plasma of mice was determined by ELISA.

RESULTS

Surface Modification of Islets With PEG-Lipid

We previously studied the modification of islet surfaces with amphiphilic polymers such as PEG-conjugated phospholipid and poly(vinyl alcohol) with alkyl side chains for the improvement of graft survival (*10–14*). Our method is promising for intraportal transplantation of islet because no damage to cell function was observed and no volume increase of islets was detected after modification of islet surface.

This study used amphiphilic PEG-lipid (shown in Fig. 1a) for modification of islet surfaces and predicted that the surface modification with PEG could suppress the inflammatory and coagulation reactions that mediate islet loss immediately after intraportal transplantation. The maleimide group at the end of the PEG chain can be used to immobilize bioactive molecules to the islet surface. Our aim was to evaluate the efficacy of PEG on islet surfaces to improve graft survival; therefore, the maleimide group was deactivated by cystein before surface modification of islets. Islets were treated with a mixture of FITC-labeled PEG-lipid and PEG-lipid, and clearly detectable fluorescence was observed at the

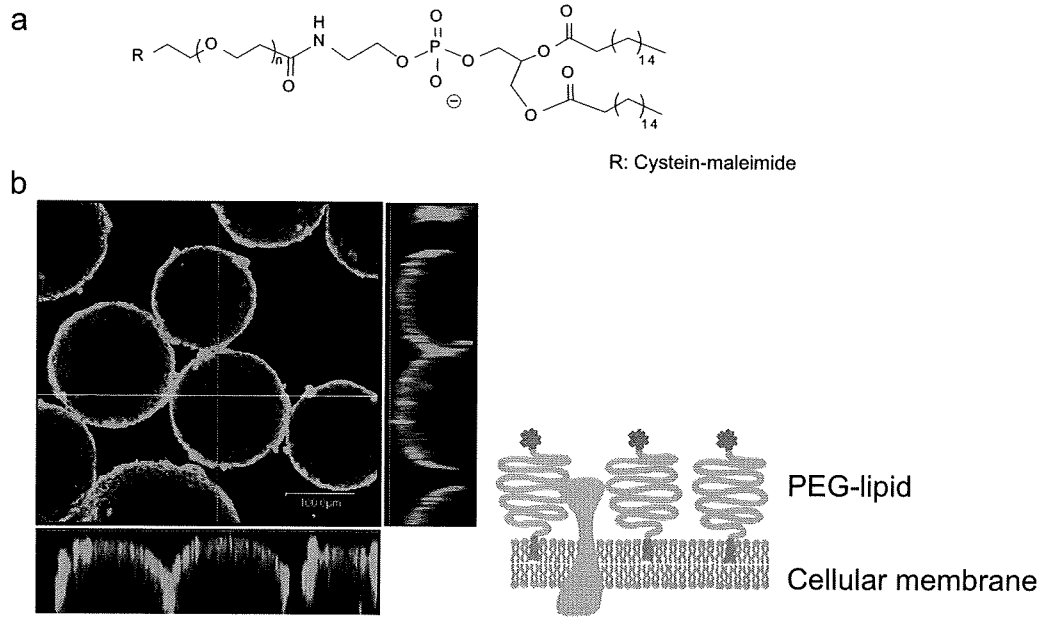

FIGURE 1. (a) Chemical structure of poly(ethylene glycol) (PEG)-conjugated dipalmitoyl-*sn*-glycerol-3-phosphatidylethanolamine (PEG-lipid). (b) Confocal laser scanning images of fluorescein isothiocyanate-PEG-lipid-modified islets from hamsters. PEG-lipid can be introduced on the surface of islets by hydrophobic interaction. The hydrophobic lipid portion of PEG-lipid was spontaneously incorporated into the lipid bilayer membrane of islets.

periphery of islets, indicating that the PEG chain was incorporated on the surface of islets (Fig. 1b). Furthermore, the hydrophobic lipid portion of PEG-lipid was observed to be spontaneously incorporated into the lipid bilayer membrane of islets. These results indicate that PEG-lipid can form a layer on the islet surface without apparent damage to islet morphology.

Insulin Secretion by Glucose Stimulation

Next, we examined the ability of PEG-islets to control insulin release in response to glucose level changes using a glucose stimulation test; the results are summarized in Figure 2. No significant difference in insulin release was observed between control islets and PEG-islets. When the glucose concentration was increased from 0.1 to 0.3 g/dL, both control and PEG-islets increased insulin release compared with basal levels. When islets were reexposed to 0.1 g/dL, insulin release returned to basal levels. These results indicate that the PEG-lipid surface modification did not affect the ability of islets to regulate and release insulin.

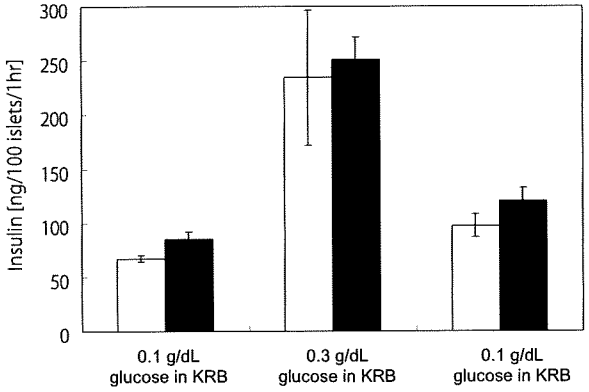

FIGURE 2. Assessment of insulin release in glucose stimulated islets. The amounts of insulin secreted from poly(ethylene glycol) islets (*black bars*) and control bare islets (*white bars*) in response to glucose concentration changes (0.1, 0.3, and 0.1 g/dL) were determined by ELISA. Results are expressed as mean±SD; n=3. KRB, Krebs-Ringer buffer solution.

Intraportal Transplantation Into Liver of STZ-Induced Diabetic Mice

We next evaluated the efficacy of PEG-modification on the improvement of graft survival immediately after intraportal transplantation. Hamster islets were transplanted into the livers of STZ-induced diabetic mice through the portal vein. This transplantation is a concordant xenotransplantation model. Recipient mice were not treated with immunosuppressive therapy. Figure 3 shows changes in nonfasting blood glucose levels of recipient mice after intraportal transplantation of 500 or 1000 bare islets or PEG-islets. In this xenotransplantation model, the blood glucose level normalized after transplantation of islets; however, levels returned to hyperglycemic levels around 7 days because the grafts were lost by immune rejection in recipient mice. In the mice transplanted with 500 islets (Fig. 3a), the blood glucose levels in all mice except one were not normalized 1 day after transplantation. Normoglycemia was achieved in four of nine mice at 3 days, and blood glucose levels were maintained for several days. At 7 days posttransplantation, most mice returned to hyperglycemic states. These results indicated that induction of islet cell damage occurred immediately after transplantation into the liver, and some of islets were destroyed, resulting in loss of function. The blood glucose level could not be properly regulated by the remaining islets during the early phase after posttransplantation. On transplantation of 1000 islets (Fig. 3b), normoglycemia was achieved in all mice at 2 days and continued until grafts were rejected (around 7 days). In contrast, when 500 PEG-islets were transplanted, five of six mice were normoglycemic at 2 days, and this was maintained

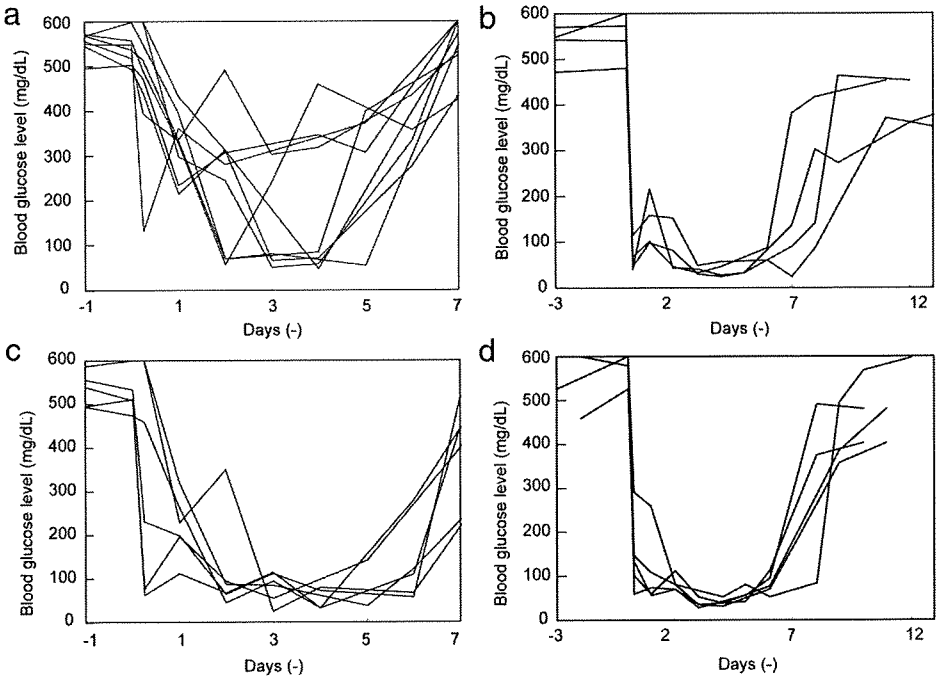

FIGURE 3. Changes in nonfasting blood glucose levels of streptozotocin-induced diabetic Balb/c mice after intraportal transplantation of (a) 500 control islets (n=9), (b) 1000 control islets (n=4), (c) 500 poly(ethylene glycol) islets (n=6), or (d) 1000 poly(ethylene glycol) islets (n=5). Graft failure was defined as two consecutive plasma glucose determinations ≥200 mg/dL.

until the graft was rejected (around 7 days). On transplantation of 1000 PEG-islets, all mice were normoglycemic at 2 days, and this was also maintained until the graft was rejected (around 7 days). The period of graft survival in recipients of control and PEG-islets transplantation are summarized in Table 1. We found a significant difference between the period of graft survival on transplantation of 500 control and PEG-islets. These results indicate that the islet damage that occurs immediately after transplantation into the liver could be suppressed by surface modification of islets with PEG-lipids. The period of graft survival was prolonged, although the immune rejection reaction could not be suppressed. On the other hand, we did not observe any significant difference in graft survival between recipients of 1000 control and PEG-islets.

TABLE 1. Graft survival days after transplantation

	Number	Graft survival days	Mean±SD	
Control islets	500	0, 0, 0, 1, 2, 3, 3, 3, 4	1.8±1.6	$P<0.01$
	1000	6, 7, 8, 9	7.5±1.7	
PEG-islets	500	4, 4, 5, 5, 6, 6	5.0±0.9	
	1000	6, 6, 6, 7, 7	6.3±0.3	

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

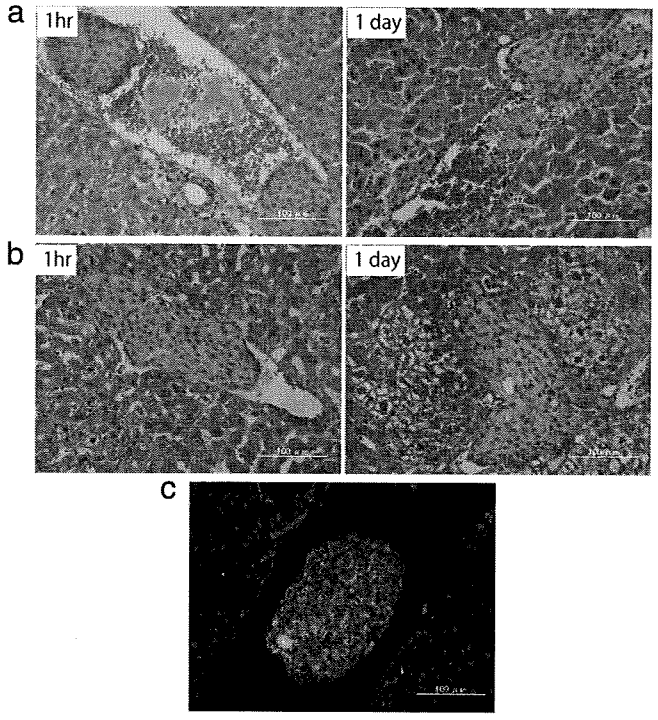

FIGURE 4. Histochemical analysis of (a) control islets and (b) poly(ethylene glycol) islets transplanted into the liver of diabetic mice through the portal vein by hematoxylin-eosin staining, and (c) poly(ethylene glycol) islets by insulin staining. These grafts were retrieved at 1 hr and 1 day postintraportal transplantation. The graft obtained 1 hr after transplantation was also stained for insulin and the nucleus (Hoechst 33342).

We speculate that mice transplanted with 500 control islets did not receive sufficient islets because most were destroyed after transplantation, and the blood glucose levels could not be controlled. On the other hand, in mice transplanted with 500 PEG-islets, there were sufficient islets remaining, as cell damage to islets could be suppressed by surface modification with PEG-lipid; furthermore, blood glucose levels could be normalized, indicating the improvement of graft survival. For mice transplanted with 1000 control islets, there were sufficient islets remaining to control blood glucose levels, despite the loss of some islets. These results suggested that it is possible to suppress complement activation and blood coagulation-mediated inflammatory reactions that induce cell damage to islets immediately after transplantation by modification of islet surfaces with PEG-lipids; this approach, however, does not suppress immune rejection reactions.

Livers were retrieved from recipient mice after transplantation of control or PEG-islets through the portal vein at 1 hr and 1 day and subjected to histochemical analyses. After control islet transplantation at 1 hr (Fig. 4a), islets were damaged and destroyed in the blood vessel of the liver. Aggregation of red blood cells was observed around destroyed islets, indicating platelet aggregation and blood coagulation on the surface of islets. Cell damage to some islets was also observed after transplantation for 1 day (Fig. 4a). However, in livers of mice transplanted with PEG-islets, most islets were not damaged and remained intact in blood vessels of liver after transplantation for 1 hr (Fig. 4b and c). The same results were observed 1 day after transplantation. These histochemical analyses support the finding that the period of graft survival is prolonged after transplantation of PEG-islets.

Determination of Insulin Levels in Blood After Intraportal Transplantation of PEG-Islets

To determine plasma insulin levels, blood was taken from the subclavian vein 15, 30, and 60 min after intraportal transplantation of control and PEG-islets. When islets undergo cell damage, large amounts of insulin from the islets are released into the blood and the blood insulin levels transiently

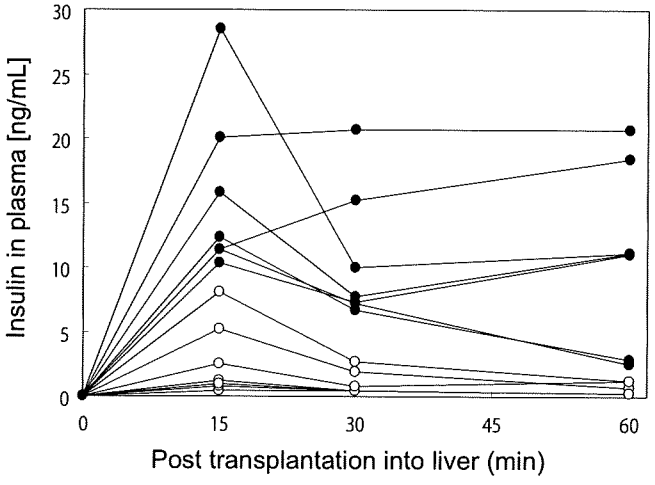

FIGURE 5. Changes in the blood insulin levels of diabetic mice after intraportal transplantation of control islets (*closed circles*, n=7) or PEG islets (*open circles*, n=7). The blood insulin levels were determined by ELISA.

increase (*9*). Therefore, the degree of cell damage to islets can be linked to the blood insulin level. In mice transplanted with control islets, high levels of insulin (~27 ng/mL) were detected 15 min after transplantation (Fig. 5). The insulin was still observed at 30 and 60 min, although the amount of released insulin gradually reduced. These results indicated that islets were damaged, resulting in the release of large amounts insulin into the blood immediately after transplantation. On the other hand, in mice transplanted with PEG-islets, the release of insulin was considerably suppressed immediately after transplantation (~7 ng/mL), indicating that islet cell damage was suppressed by PEG-lipid modification. Thus, this indicates the possibility to improve the biocompatibility of islets by surface modification with PEG-lipids.

DISCUSSION

Several groups have attempted to improve graft survival of islets by modification of islet surfaces (*8*, *9*, *16*, *17*). Byon et al. reported improved graft survival of islets on covalent bonding of PEG to cell membrane proteins and low dose of immunosuppressive therapy by transplantation under the kidney capsule. However, it is difficult to expect in the current clinical setting that surface modification of islets by PEG would improve transplantation under kidney capsules (*18*), as clinical islet transplantation to the liver is performed through the portal vein, and islets are exposed to blood components. On the other hand, when islets were transplanted under kidney capsules, cells are not exposed to blood components. The periods of graft survival are different in transplantation between these sites (*18*). When islets are exposed to fresh blood in the portal vein, the blood coagulation and complement systems are activated, and chemotactic factors, tissue factor, chemokines, and other inflammatory mediators are released during the early phase of transplantation. These factors are induced by instant blood-mediated inflammatory reactions, resulting in graft loss and reduction of graft survival in the liver. Therefore, for future clinical islet transplantation, it is necessary to examine the efficacy of surface modification of islets by transplantation into liver. Several reports have examined the effect on transplantation success when transplanting surface-modified islets into livers of diabetic animals (*8*, *9*). Contreras et al. (*8*) reported that the graft survival of porcine islets in livers of NOD-SCID mice was improved by the transfection of an apoptosis-suppressing gene and covalent PEG-surface modification. Nilsson and coworkers (*9*) reported that the graft survival of porcine islets could be improved by modifying the surface of islets with heparin and found that the heparin coating suppressed blood coagulation and activation of complement systems.

This study modified the surface of islets with amphiphilic PEG-lipid and examined the ability of the surface modification to improve graft survival in the liver. Recently, we also immobilized bioactive molecules such as fibrinolytic urokinase using amphiphilic polymers to improve graft survival of islets in the liver. We have predicted that the surface modification with PEG and urokinase could suppress the inflammatory and coagulation reactions that mediate islet loss immediately after intraportal transplantation. PEG-lipid was spontaneously anchored to the lipid bilayer of cell membrane through hydrophobic interactions without cytotoxicity. Therefore, it is possible for a thin PEG layer to form on the islet surface without volume increase. PEG-lipid has been used for the surface modification of liposomes to improve biocompatibility and prolong the circulation time in vivo in drug delivery systems (*19*, *20*). Complement activation and platelet aggregation are suppressed by the surface modification of liposome with PEG-lipid (*21*, *22*). Our group studied the interaction between serum components and PEG-immobilized substrate by surface plasmon resonance and did not find activation of complement on the PEG-immobilized substrate (*23*). In this study, PEG-lipid modified hamster islets were transplanted into livers of STZ-induced diabetic mice through the portal vein. We predicted that complement activation and platelet aggregation could be suppressed in the early phase using the PEG-lipid modified surface, as seen for PEG-liposomes and PEG-immobilized substrate. The graft survival of PEG-islets was significantly prolonged compared with bare islets in livers of diabetic mice (Fig. 3). Reduction of blood insulin level within 60 min after transplantation suggests that the cell damage observed immediately after transplantation could be suppressed by surface modification with PEG (Fig. 5). Platelet activation and complement system activation is believed to be suppressed using islets modified with PEG-lipids, as seen with PEG-liposomes and PEG-immobilized substrate. However, it was not possible to suppress immunerejection reactions in recipient mice during later phases. Future studies should seek to improve stable and thick polymer layers on the islet surface and reveal the mechanism for prevention of graft loss with PEG modification in vivo and in vitro.

CONCLUSIONS

A thin PEG layer could form on the surface of islets using PEG-lipids without volume increase and cytotoxicity. Blood coagulation and activation of complement systems, which were induced immediately after intraportal transplantation, could be suppressed using the PEG-lipid modified islets in transplantation, and usually observed islet damage during transplantation was suppressed. Furthermore, the period of graft survival was significantly improved. Together these findings suggest that our approach described here for the improvement of graft survival will be useful in the clinical setting.

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Control of cell attachment through polyDNA hybridization

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ABSTRACT

Cell–cell interactions play vital roles in embryo development and in homeostasis maintenance. Such interactions must be stringently controlled for cell-based tissue engineering and regenerative medicine therapies, and methods for studying and controlling cell–cell interactions are being developed using both biomedical and engineering approaches. In this study, we prepared amphiphilic PEG-lipid polymers that were attached to polyDNA with specific sequences. Incubation of cells with the polyDNA–PEG-lipid conjugate transferred some of the polyDNA to the cells' surfaces. Similarly, polyDNA–PEG-lipid conjugate using polyDNA with a complementary sequence was introduced to the surfaces of other cells or to a substrate surface. Cell–cell or cell–substrate attachments were subsequently mediated via hybridization between the two complementary polyDNAs and monitored using fluorescence microscopy.

1. Introduction

In the past decade, therapeutic devices containing living cells or tissues have been studied extensively for tissue engineering and regenerative medicine applications. Stem cells, including embryonic stem (ES) cells, somatic stem cells, and induced pluripotent stem (iPS) cells, have been identified and studied [1–3] that show promise for treatment of diseases such as type I diabetes, Parkinson's, Alzheimer's, ALS, and Huntington's disease [4–11]. Experimental manipulation of cell–cell interactions is a valuable method for inducing differentiation of stem cells for use in cell-based therapies. In addition, the differentiated cells can be manipulated further for use in regenerating tissues or organs. Cell–cell interactions must be tightly controlled for generating cell-type-specific tissues or organs. Cell–cell interactions are also used to develop pluripotent stem cells themselves. It was reported recently that somatic cells could be transformed into pluripotent stem cells by fusion with ES cells [12]. In this method, somatic cells and ES cell attachments formed first, and attachment was followed by induced cell fusion.

Cell–cell interactions are also very important in embryo development and in the maintenance of homeostasis. Methods for studying and controlling cell–cell interactions are currently being developed using both biomedical and engineering approaches. Our group has studied the surface modification of living cells using amphiphilic polymers such as PEG-conjugated phospholipid (PEG-lipid) derivatives [13–19]. Specifically, our previous efforts were directed towards modification of cell surfaces and islets of Langerhans (islets) by introducing functional groups and polymers for improving graft survival after transplantation. Recently, immobilization of cells to the surface of islets using PEG-lipid and a biotin/streptavidin reaction resulted in encapsulation of the whole islet surface with layers of cells [19]. It seemed possible to use this method to induce cells to attach to a substrate. Although the biotin/streptavidin reaction is well characterized and is used frequently in biological studies, it has some disadvantages. Specifically, streptavidin is derived from bacteria and is a potent antigen in humans; further, the biotin/streptavidin association is so strong that it is difficult to be dissociated.

In the present study, we employed DNA hybridization rather than the biotin/streptavidin reaction as a novel method for inducing cell–cell attachment and cell immobilization on a substrate. We used PEG-lipid, which is an amphiphilic polymer, as a carrier for polyDNA with a specific sequence. Cells treated with the polyDNA–PEG-lipid conjugate incorporated the lipid (and thus the polyDNA) onto the cell surface. PolyDNA with the complementary sequence was similarly transferred onto the surface or other cells or onto a substrate. Cell–cell or cell–substrate attachments were subsequently induced via hybridization between the two complementary polyDNAs.

2. Materials and methods

2.1. Materials

α-*N*-Hydroxysuccinimidyl-ω-maleimidyl poly(ethylene glycol) (NHS-PEG-Mal, MW: 5000) was from Nektar Therapeutics (San Carlos, CA, USA). 1,2-dipalmitoyl-*sn*-

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Table 1
Sequence of DNA for cell surface modification.

	5′–3′
polyA20	HS – AAA AAA AAA AAA AAA AAA AA
polyT20	HS – TTT TTT TTT TTT TTT TTT TT
SeqA	HS – TGC GGA TAA CAA TTT CAC ACA
SeqA′	HS – TGT GTG AAA TTG TTA TCC GCA
SeqB	HS – TAG TAT TCA ACA TTT CCG TGT
SeqB′	HS – ACA CGG AAA TGT TGA ATA CTA

glycerol-3-phosphatidylethanolamine (DPPE) was from NOF Corporation (Tokyo, Japan). Dichloromethane, triethylamine, and diethyl ether was from Nacalai Tesque (Kyoto, Japan). Hanks' balanced salt solution (HBSS), minimum essential medium (MEM), and RPMI-1640 medium were from Invitrogen Co. (Carlsbad, CA, USA). Fetal bovine serum (FBS) was from Equitech-Bio, Inc. (TX, USA), and phosphate-buffered saline (PBS) was from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). PKH67 Green Fluorescent Cell Linker Kit (PKH green) and PKH26 Red Fluorescent Cell Linker Kit (PKH red) were from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). *n*-Hexadecyl mercaptan was from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Glass plates (22 mm × 26 mm; thickness: 0.12–0.17 mm) were from Matsunami Glass Ind., Ltd (Osaka, Japan). Dithiothreitol (DTT) was from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

2.2. Synthesis of DNA-conjugated PEG-phospholipid (polyDNA–PEG-lipid)

Mal-PEG-lipid was synthesized by combining NHS-PEG-Mal (180 mg), triethylamine (50 μL), and DPPE (20 mg) with dichloromethane and stirring for 36 h at room temperature (RT) [14]. After precipitation with diethyl ether, Mal-PEG-lipid was obtained as a white powder (190 mg, 80% yield). ^{1}H NMR analysis ($CDCl_3$, 400 MHz, δ 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 DNA sequences used in this study are listed in Table 1. DNA was synthesized by Sigma–Aldrich Chemical Co. DNA-SH was prepared by reduction of the disulfide bond with DTT according to the manufacturer's instructions. A PBS solution of DNA-SH (1.0 mg) was mixed with Mal-PEG-lipid (5.0 mg) in PBS for 24 h at RT to prepare polyDNA–PEG-lipid. PolyDNA–PEG-lipid (500 μg/mL in PBS) was used for surface modification of cells without purification.

2.3. Cell cultures

Two cell lines, CCRF-CEM cells (a human T cell lymphoblast-like cell line) and HEK293 cells (a human embryonic kidney cell line) were obtained from the Health Science Research Resources Bank (Osaka, Japan). Suspension culture of CCRF-CEM cells was performed in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Invitrogen) at 37 °C under 5% CO_2. HEK293 cells that stably expressed enhanced green fluorescence protein (EGFP) (GFP-HEK) were the kind gift of Dr. K. Kato (Institute for Frontier Medical Sciences, Kyoto University). The GFP-HEK cells were maintained in MEM supplemented with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin.

2.4. Surface modification of cells with polyDNA–PEG-lipid and co-incubation of differentially modified cells

For visualization under a fluorescence microscope, CCRF-CEM cells were labeled with PKH red or PKH green according to the manufacturer's instructions. To exchange the culture medium, CCRF-CEM or GFP-HEK cells (4×10^6 cells) were washed twice with HBSS and collected by centrifugation (180g, 5 min, 25 °C). After the addition of polyDNA–PEG-lipid solution (50 μL, 500 μg/mL in PBS) to the cell suspension, cells were incubated for 30 min at RT with gentle agitation. The cells were then suspended in 10 mL HBSS, collected by centrifugation (180g, 5 min, 25 °C), washed with another 10 mL HBSS, and re-centrifuged to obtain polyDNA–PEG-lipid-modified cells.

After cells were treated with polyA–PEG-lipid or polyT–PEG-lipid, the polyDNA–PEG-lipid-modified cells were mixed together in culture medium with the following ratios of polyA-cells:polyT-cells: 10:1, 4:1, 2:1, and 1:1. The cells were incubated with rotation at 100 rpm for 1 h at RT, followed by incubation at 37 °C under 5% CO_2. The cells were observed over time using a confocal laser scanning microscope (FLUOVIEW FV500, Olympus, Tokyo, Japan) and a phase-contrast microscope (IX7, Olympus Optical Co. Ltd., Tokyo, Japan).

2.5. Immobilization of polyDNA–PEG-lipid modified cells to patterned substrates

SeqA-conjugated PEG-lipid and SeqB-conjugated PEG-lipid were used for cell surface modification. For testing immobilization of the modified cells, substrate surfaces were modified using SeqA′ and SeqB′, the sequences complementary to SeqA and SeqB.

Glass plates were cleaned with a piranha solution (7:3 mixture of concentrated sulfuric acid and 30% hydrogen peroxide solution), washed 3× with Milli-Q water, and stored in a 2-propanol solution. For experiments, glass plates were mounted on a rotation stage in a metal vapor deposition apparatus (V-KS200, Osaka Vacuum Instruments, Osaka, Japan). A 1.0-nm chromium layer was deposited on the glass, followed by deposition of a 19-nm gold layer. The resulting glass plates coated with a thin layer of gold were immersed in an ethanol solution of *n*-hexadecyl mercaptan (1 mM) to produce a surface with SAM-carrying methyl groups (CH_3-SAM). The CH_3-SAM surface was irradiated with an ultraviolet (UV) light at 180 mW/cm^2 using an Optical ModuleX (SX-UI 501HQ, Ushio, Inc., Tokyo) equipped with a super-high-pressure mercury lamp (Ushio, Inc.) through a photomask with an array of transparent 1- or 2-mm circular dots in ambient air for 4 h. The plates were washed with ethanol to remove photodegradation products. A PBS solution of DNA-SH (600 μg/mL, SeqA′ and SeqB′), was applied to the UV-irradiated spots by manual pipetting and allowed to incubate for 2 h at RT. The substrate-coated glass plate was washed with HBSS before use.

In the first series of experiments, SeqA-PEG-lipid modified CCRF-CEM cells (SeqA-PEG-cells) and SeqB-PEG-lipid modified CCRF-CEM cells (SeqB-PEG-cells) were mixed at the following ratios: 4:1, 2:1, 1:1, 2:1, and 4:1. The cell suspensions were applied to UV-irradiated spots that had been incubated with a 1:1 mixture of SeqA′ and SeqB′ (see above); cells were incubated on the immobilized-DNA surface for 10 min at RT. In a second series of experiments, the UV-irradiated spots were incubated with SeqA′:SeqB′ at the following molar ratios: 4:1, 2:1, 1:1, 2:1, and 4:1. A 1:1 mixture of SeqA-PEG-cells and SeqB-PEG-cells was then applied to the UV-irradiated spots containing immobilized DNA. After washing with HBSS, cells attached to the substrate were observed using an upright fluorescence microscope (BX51, Olympus, Tokyo, Japan) and a stereomicroscope (MZF LIII, Leica, Solms, Germany). The number of attached cells was analyzed using ImageJ software (NIH, Bethesda, MD, USA).

An inhibition assay was also performed using a solution of SeqA′ (200 μg/mL) that was added to the mixture of SeqA-PEG-cells and SeqB-PEG-cells. After incubation for 30 min, the mixture was applied to the SeqA′ and SeqB′-immobilized substrate and incubated for 10 min at RT. After washing with HBSS, the cells attached to the substrate were observed using an upright fluorescence microscope.

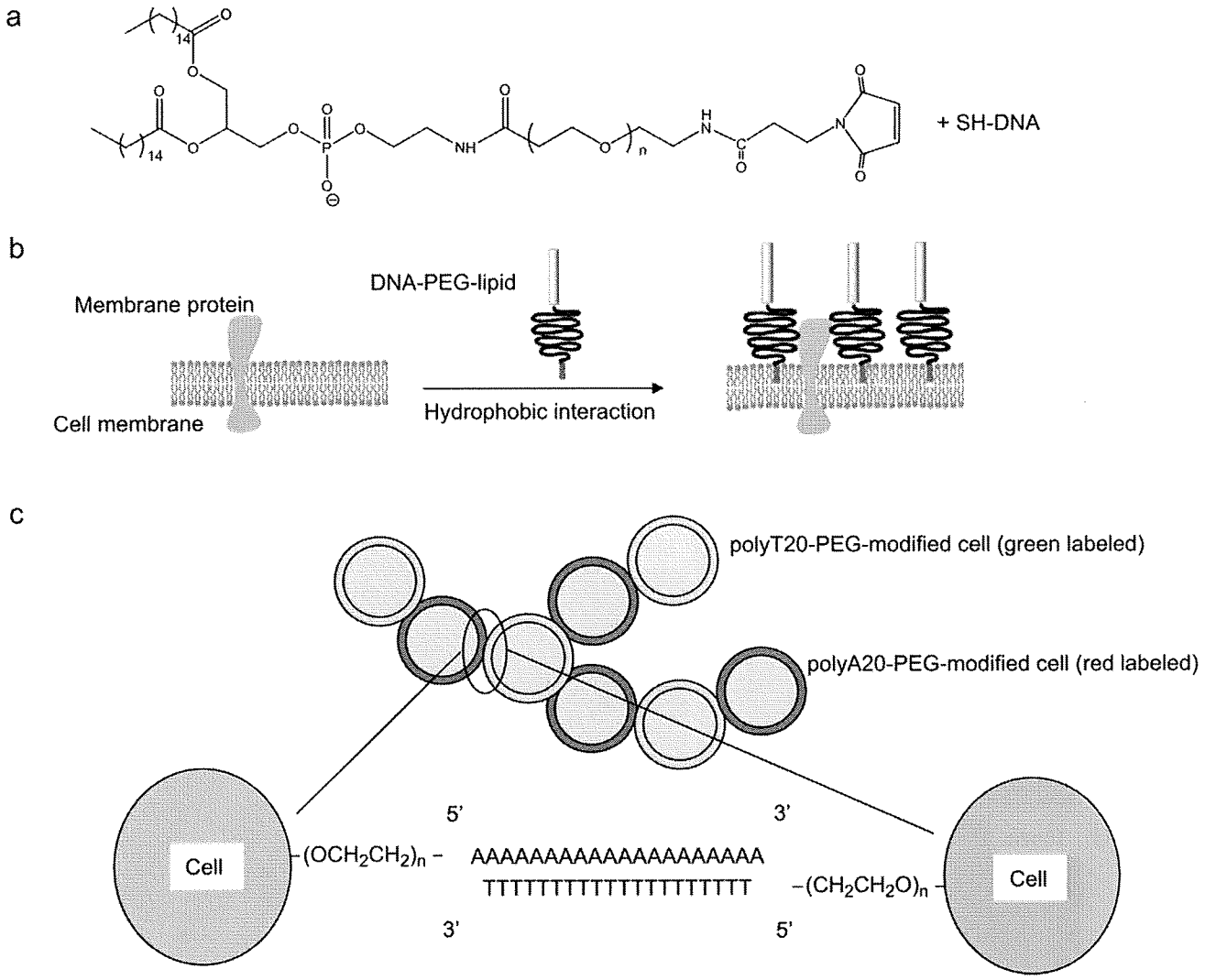
Substrates for cell attachment were also prepared using a contact printing technique. Poly(dimethylsiloxane) (PDMS) stamps were prepared as follows: A ledge pattern was fabricated on a PDMS surface using a laser beam machine (VLS2.30, Universal Laser Systems, Inc., Scottsdale, AZ, USA): The pattern consisted of unidirectional ledges (1 mm × 1 mm × 10 mm) with 1-mm intervals between ledges. The ledge surfaces on the stamps were coated with a solution of SeqA′ or SeqB′ DNA-SH (600 μg/mL) and applied to the gold-layered glass plates. A second stamp coated with a solution of SeqA′ or SeqB′ DNA-SH was applied to the surface perpendicular to the previous ledge design. The glass plate sat at RT for 2 h to dry. The glass plate was then immersed in an ethanol solution of *n*-hexadecyl mercaptan for blocking with CH_3-SAM and washed with ethanol and Milli-Q water. A 1:1 mixture of SeqA-PEG-cells and SeqB-PEG-cells were applied onto the patterned substrate and incubated for 10 min at RT with gentle agitation. After washing with HBSS, cells attached to the glass plate were observed using an upright fluorescence microscope.

3. Results

3.1. Intercellular attachment through hybridization of complementary polyDNA–PEG-lipid conjugates

Scheme 1 shows how cells carrying complementary polyDNA–PEG-lipid conjugates were tested for intracellular attachment. polyDNA–PEG-lipids were synthesized using a thiol/maleimide reaction between Mal-PEG-lipid and DNA-SH in which the SH group was introduced at the 5′-end of the DNA sequence. The DNA sequences used in this study are listed in Table 1. polyDNA–PEG-lipids carrying complementary sequences were prepared: polyA20 and polyT20, SeqA and SeqA′, SeqB and SeqB′. Our previous studies demonstrated that amphiphilic PEG-lipids are spontaneously incorporated into the cell membrane's lipid bilayer through hydrophobic interactions and that this incorporation has no cytotoxic effects [13–16,18,19]. We further showed that polyDNA could be introduced onto the cell surface using a PEG-lipid (Scheme 1b). The strategy in the present study was to mediate cell–cell interactions by hybridization between complementary DNA sequences that were incorporated into the cells' outer membranes (Scheme 1c).

Incorporation of polyA20–PEG-lipid into the cell membrane and its ability to hybridize with FITC-labeled polyT20 was examined



Scheme 1. (a) Synthesis of DNA-conjugated PEG-DPPE (polyDNA–PEG-lipid) from maleimide–PEG-lipid and DNA-SH. (b) Schematic illustration of the interaction between polyDNA–PEG-lipid and the lipid bilayer comprising the outer cell membrane. The polyDNA–PEG-lipid inserts into the cell membrane due to hydrophobic interactions between the acyl chain and the lipid bilayer. (c) Schematic illustration of cell–cell attachment through DNA hybridization between complementary polyDNA–PEG-lipids incorporated into the outer cell membranes.

first. A solution of polyA20–PEG-lipid was added to CCRF-CEM cells; after incubation, the cells were washed to remove unincorporated lipid, FITC-labeled polyT20 was added, and cells were observed using a confocal laser scanning microscope. As shown in Fig. 1a the FITC fluorescence was observed at the periphery of all cells, indicating that polyA20–PEG-lipids were incorporated into the outer cell membrane and that FITC-labeled polyT20 hybridized with the incorporated polyA20 DNA. When FITC-labeled polyA20 was added to polyA20–PEG-lipid modified cells, no fluorescence was observed on the cells. These results indicated that FITC-labeled polyT20 hybridized specifically with polyA20–PEG-lipids on the cell surface.

Intercellular attachments could also be mediated by hybridization between polyA20 and polyT20, as shown in Fig. 1c. CCRF-CEM cells labeled with PKH red were treated with polyA20–PEG-lipids (polyA20–PEG cells) and CCRF-CEM cells labeled with PKH green were treated with polyT20–PEG-lipids (polyT20–PEG-cells). Red polyA20–PEG-cells and green polyT20–PEG-cells were mixed at ratio of 1:1 and observed over time by a confocal laser scanning microscope (Fig. 1c). At 15 min after mixing, polyA20–PEG cells (red) and polyT20–PEG-cells (green) were attached to each other, with several cells attached in a linear fashion. At 60 min, even more cells had attached to each other. At 3 h, the linear cell aggregates had gathered to form clumps of cells. At 6 h, the cellular clumps were still present in the culture medium. As a control experiment, PKH red- and PKH green-labeled cells with no polyDNA–PEG-lipid treatment were mixed. These cells showed no attachment to each other (Fig. 1e). In addition, there was no self attachment between polyT20–PEG-cells. These results clearly showed that the attachment of different cells could be induced by hybridization between polyA20 DNA and polyT20 DNA on the cell surfaces. The ratio of the number of attachments between polyA20–PEG-cells and polyT20–PEG-cells to the total number of attachments for all cells was approximately 1 at 15 and 60 min of incubation, indicating the alternating attachment of polyA20–PEG-cells and polyT20–PEG-cells. At 3 h, the ratio had decreased to approximately 0.6, indicating that larger aggregates of cells had formed. Cell–cell attachments could also be induced between polyA20–PEG-lipid modified CCRF-CEM cells (red) and polyT20–PEG-lipid modified GFP-HEK cells (green), as seen in Fig. 1d). In contrast, no cell–cell attachments

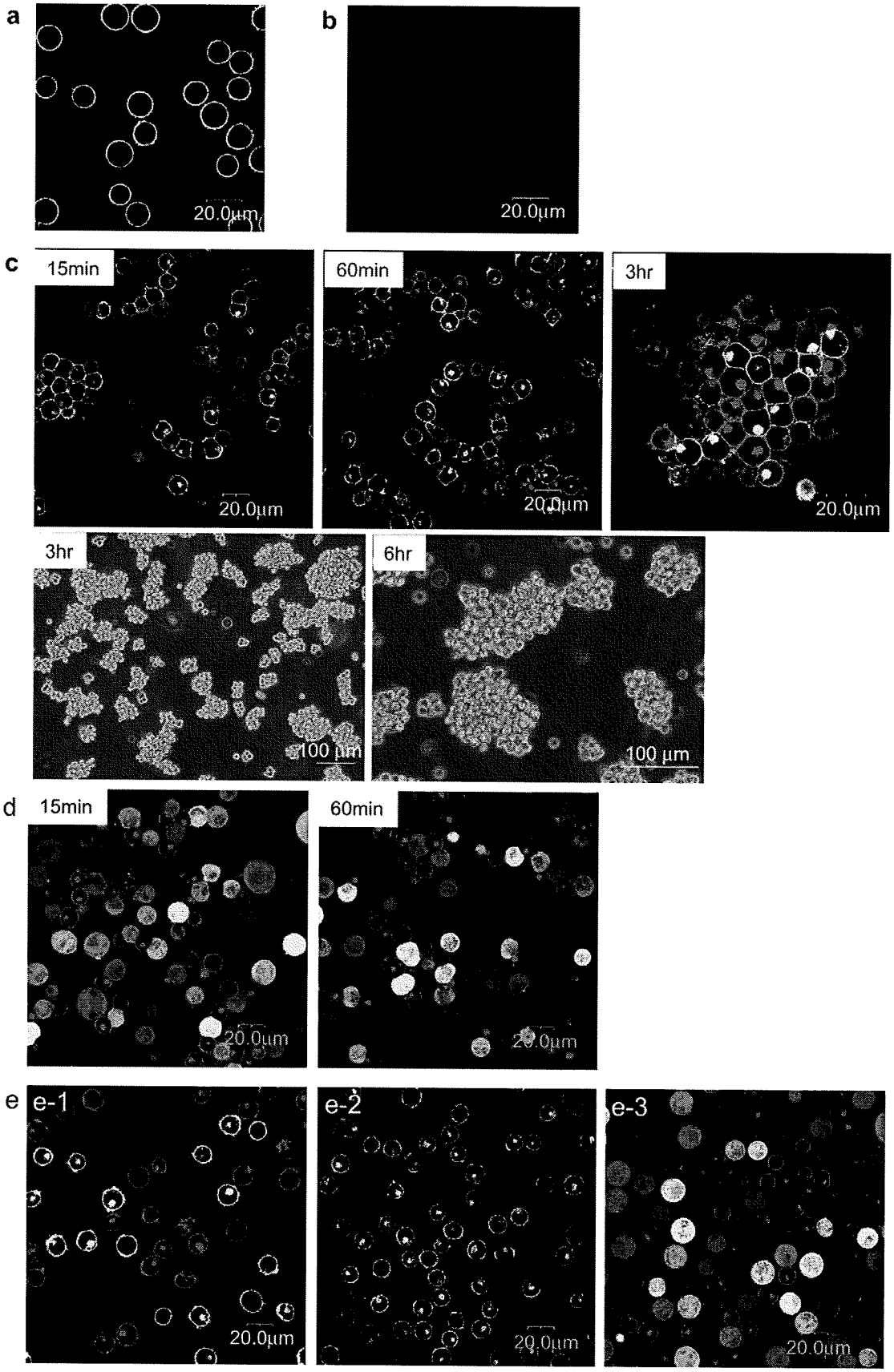


Fig. 1. Cell–cell attachment via DNA hybridization between complementary polyDNA–PEG-lipids on cell surfaces. CCRF-CEM cells incorporated polyA20–PEG-lipid into the outer cell membranes. Cells were observed by a confocal laser scanning microscope after polyA20–PEG-lipid modified CCRF-CEM cells were further treated with (a): FITC-labeled polyT20 and (b): FITC-labeled polyA20. (c): Cell–cell attachment between polyA20–PEG-lipid modified CCRF-CEM cells labeled with PKH red and polyT20–PEG-lipid modified CCRF-CEM cells labeled with PKH green in culture medium (cells were mixed in a 1:1 ratio). Cells were observed over time using a confocal laser scanning microscope and a phase-contrast microscope. (d): Cell–cell attachment between polyA20–PEG-lipid modified CCRF-CEM cells and polyT20–PEG-lipid modified GFP-HEK293 cells (cells were mixed in a 1:1 ratio). (e): Control experiments for cell–cell attachment by surface modification with polyDNA–PEG-lipid. (e–1): A mixture of CCRF-CEM cells labeled with PKH red and CCRF-CEM cells labeled with PKH green (no polyDNA–PEG-lipid modification). (e–2): PolyT20–PEG-lipid modified cells. (e–3): A mixture of CCRF-CEM cells labeled with PKH green and GFP-HEK293 cells after rotation culture at 100 rpm (no polyDNA–PEG-lipid modification).