mice as a fourth group. One of these mice was killed at 41 days after transplantation for histochemical analysis.

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. at least once per week. 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.

Histochemical Analysis

Mic-islets cultured for 1 week and cryopreserved Mic-islets cultured for 1 day and 1 week were collected in conical tubes (200 capsules/tube) and washed with phosphate-buffered saline (PBS). Then, 4% paraformaldehyde in PBS was added and the samples were incubated for 12 hr at RT. Paraformaldehyde was removed and the islets were sequentially maintained in 3% and 10% sucrose in PBS for 12 hr, followed by incubation in 20% sucrose in PBS for 24 hr at RT. Then, each group of samples was embedded in Tissue-Tek for freezing (Sakura finetechnical Co. Ltd., Tokyo, Japan). The frozen specimens were sliced (6-μm thick) and permeabilized with 0.2% Triton X-100 in PBS at RT for 15 min. The samples were then treated with a 10% goat normal serum in PBS (Dako, Denmark) for 1 hr to block nonspecific binding of antibodies. The samples were then treated with 1% polyclonal guinea pig anti-insulin (Dako) in PBS containing 3% goat normal serum at 4°C overnight, followed by washing with PBS. Next, the samples were incubated with fluorescently labeled secondary antibody, 0.2% Alexa 488 goat anti-guinea pig IgG (Molecular probes, Eugene, OR) in PBS containing 3% goat normal serum at RT for 1.5 hr. Cell nuclei were counterstained with Hoechst 33342. The localization of secondary antibodies and the Hoechst dye was analyzed by fluorescence microscopy (IX71, Olympus Optical Co. Ltd., Tokyo, Japan). Samples from each block were also stained with hematoxylin-eosin.

Transplanted cryopreserved Mic-islets were removed from the peritoneal cavities at 41 days after transplantation and were processed for histochemical analyses as described above.

RESULTS

Evaluation of Cryopreserved Mic-Islets "In Vitro"

Mic-islets that had been cryopreserved and stored in liquid nitrogen for at least 2 weeks were used for in vitro analysis. Figure 1 shows phase contrast images and immunohistochemical stained images of noncryopreserved Mic-islets after 7 days in culture and cryopreserved Mic-islets after 1 day

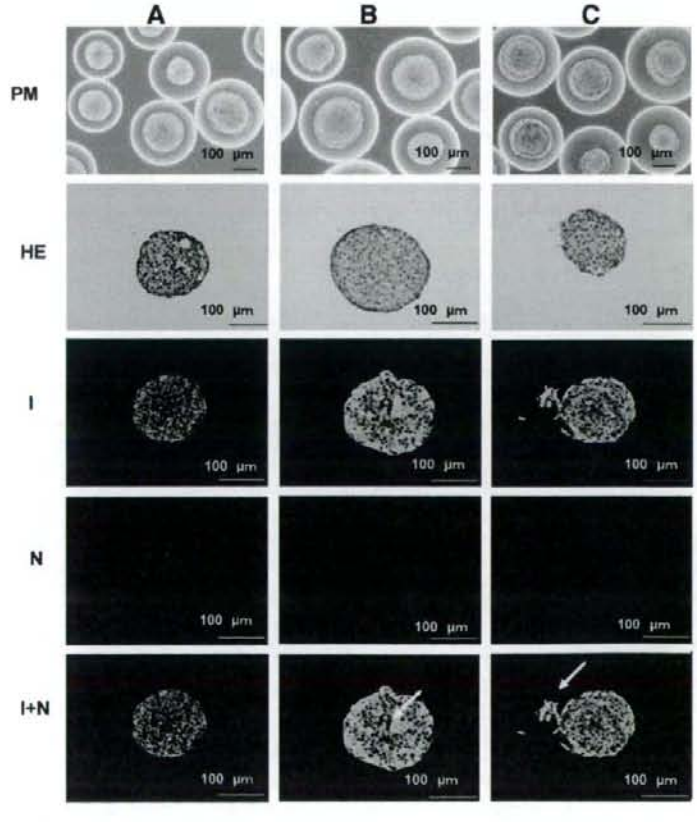

FIGURE 1. Morphology and histology of Mic-islets after cryopreservation and thawing and culture for 1 day or 1 week. (A) Mic-islets cultured for 1 week without cryopreservation as a control. Cryopreserved Mic-islets cultured for (B) 1 day and (C) 1 week after thawing. The morphology of the Mic-islets was examined by phase-contrast microscopy (PM). These samples were stained with hematoxylin-eosin (H&E), Alexa 488-labeled anti-insulin antibody (I), and with Hoechst 33342 dye for nuclear staining (N). I+N indicates the merged images from insulin and Hoechst 33342 staining.

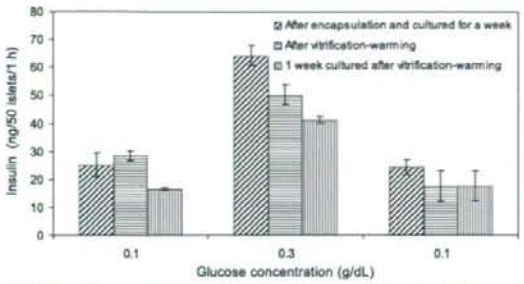

FIGURE 2. Amounts of insulin secreted by Mic-islets and cryopreserved Mic-islets in response to glucose stimulation. Cryopreserved Mic-islets were thawed, warmed, and cultured for 1 day (n=3) and 1 week (n=3). Mic-islets cultured for 1 week without cryopreservation were used as controls (n=3). Data are presented as mean±standard deviation.

and 7 days in culture. An agarose gel layer was not visible in any of the images because the section of agarose gel was peeled off of the glass slides during the staining process. There were no significant differences in the morphology of islets or agarose microcapsules between noncryopreserved and cryopreserved Mic-islets cultured for 1 day. Although some cellular deterioration was visible in the outer layer of islets after 7 days culture, most islet cells and agarose microcapsules were well preserved. Islets with and without cryopreservation were stained with an anti-insulin antibody and with Hoechst dye and the images were merged (Fig. 1). A small number of cells stained with the anti-insulin antibody but not with the Hoechst dye (arrows in Fig. 1), indicating that while most of cells in the islets were alive and expressed insulin, a few cells were damaged and died during the cryopreservation and thawing processes.

We next examined whether cryopreservation had affected the insulin secreting abilities of islets in vitro by administering a glucose challenge. Mic-islets were sequentially exposed to 0.1 g/dL, 0.3 g/dL, and 0.1 g/dL glucose in Krebs-Ringer solution for 1 hr each at 37°C. As shown in Figure 2, although Mic-islets and cryopreserved Mic-islets demonstrated the same insulin secreting ability when exposed to lower concentrations of glucose, at higher glucose concentrations cryopreserved Mic-islets secreted less insulin than the noncryopreserved Mic-islets. Cryopreserved Mic-islets cultured for 7 days released less insulin than did noncryopreserved Mic-islets at both low- and high-glucose concentrations: insulin secretion was reduced to 78% and 64% of the noncryopreserved Mic-islet levels, respectively. However, these cells did control insulin release in response to glucose concentration changes.

Evaluation of Cryopreserved Mic-Islets "In Vivo"

To study the long-term therapeutic potential of cryopreserved Mic-islets as a bioartificial pancreas in vivo, the islets were transplanted into the intraperitoneal cavities of diabetic Balb/c mice. The immunoisolative effect of agarose encapsulation was not sufficient to protect the xenogeneic hamster islets from the immune system of the recipient mice; therefore, 15-deoxysperguarin, an immunosuppressive drug, was administered to the transplant recipients daily. Figure 3 shows the changes in the nonfasting blood glucose levels of recipient mice after implantation of bare islets, Mic-islets, and cryopreserved Mic-islets (Fig. 3). When bare islets were transplanted, the blood glucose levels of all recipient mice normalized for some days. However, hyperglycemia returned quickly (Fig. 3A). When Mic-islets were transplanted, the blood glucose levels of all recipient diabetic mice returned to normal. The length of the normoglycemic period varied with the number of transplanted Mic-islets with or without cryopreservation. The average (±SD) normoglycemic periods were 53.2±16.7, 32.0±13.2, and 46.3±13.3 days for 1000 Mic-islets (n=7), and 1000 (n=5) and 2000 (n=4) cryopreserved Mic-islets, respectively. As shown in Figure 3(B), two of seven recipients of 1000 Mic-islets without cryopreservation were normoglycemic at 63 and 83 days postimplantation and the normoglycemic periods of the other five recipients ranged from 38 to 50 days. For recipients of 1000 and 2000 cryopreserved Mic-islets, the periods of normoglycemia reached 46 and 56 days, respectively (Fig. 3C–D), indicating that cryopreserved Mic-islets functioned normally in controlling the blood glucose level of recipient mice. Figure 3(F) shows the survival plots of the 1000 Mic-islets grafts and the 1000 and 2000 cryopreserved Mic-islets grafts.

These results showed that normoglycemia could be achieved by the transplantation of cryopreserved Mic-islets. The normoglycemic period of mice transplanted with 1000 Mic-islets was similar to that of recipients of 2000 cryopreserved Mic-islets. Apparently, portions of the islets were damaged during the cryopreservation process and the insulin secreting ability of the transplants islets was reduced, as shown in Figures 1 and 2.

Histochemical Analysis of Transplanted Cryopreserved Mic-Islets

Transplanted cryopreserved Mic-islets were retrieved from the peritoneal cavities of recipients of 3000 Mic-islets at 41 days posttransplantation and were subjected to immunohistochemical analysis. Figure 4 shows microscopic images of thin sections stained with hematoxylin-eosin and Alexa 488-labeled anti-insulin antibodies. Agarose capsules that had been cryopreserved in vitrification solution were consistently surrounded by adipose tissue and were slightly deformed, but remained intact. No inflammatory cell infiltration was observed around or inside the agarose capsules. Hamster islets within the capsules were isolated from mouse tissues 41 days after transplantation. Immunohistochemical analysis also showed that the Mic-islets expressed insulin. Anti-insulin-positive cell debris was present on the surface of the islets, most likely due to nonspecific insulin adsorption by dead cells within the agarose beads (arrow in Figure 4).

DISCUSSION

Our previous work (*15*) demonstrated that 55% of normal insulin secretion ability was maintained in cryopreserved Mic-islets after the vitrification-warming process and that the mechanical properties and molecular weight cut-off of the agarose hydrogel were well maintained, albeit slightly reduced. In this study, we examined the feasibility of using cryopreserved

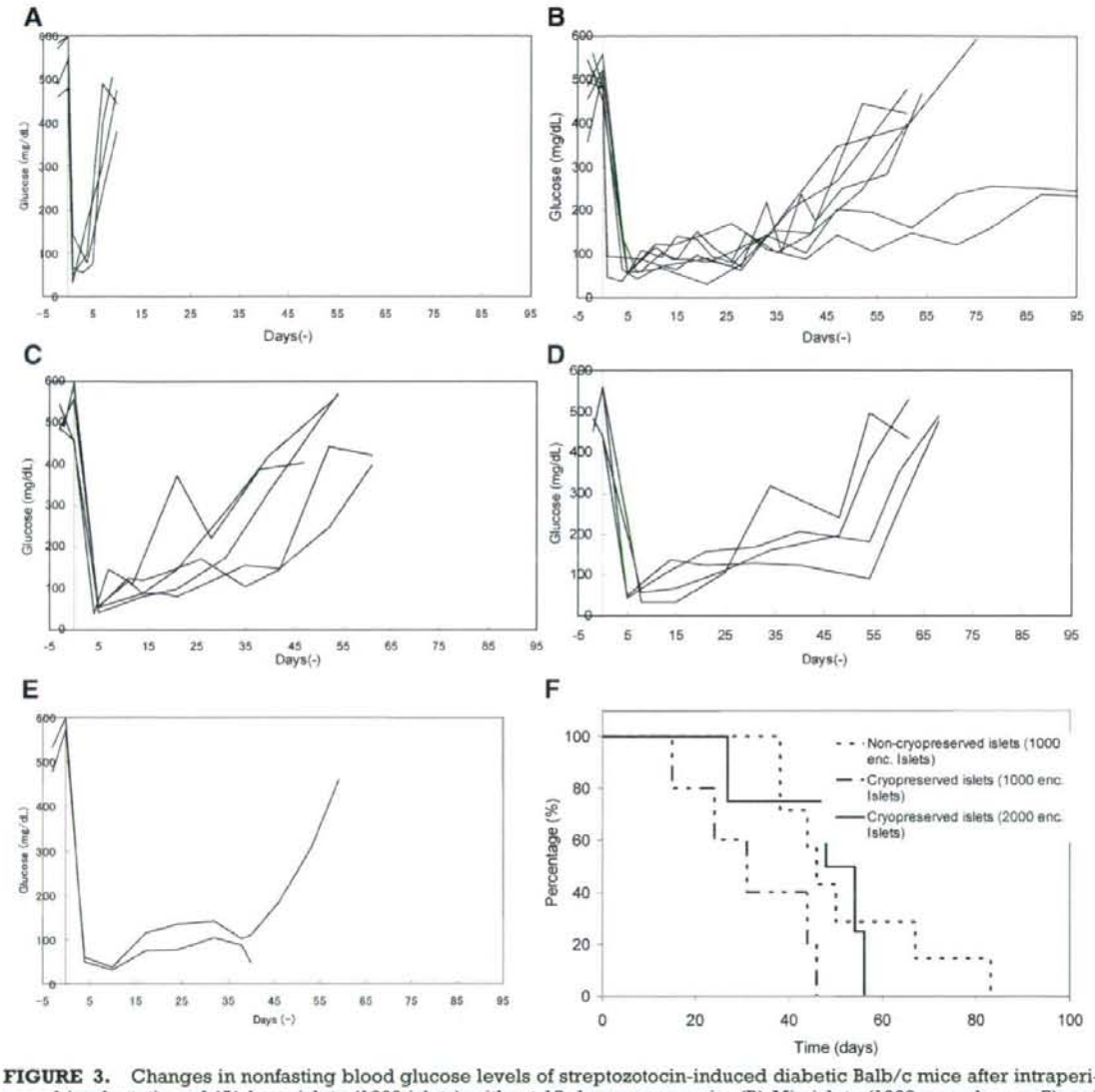

FIGURE 3. Changes in nonfasting blood glucose levels of streptozotocin-induced diabetic Balb/c mice after intraperitoneal implantation of (A) bare islets (1000 islets) without 15-deoxysperguarin, (B) Mic-islets (1000 capsules, n=7) and cryopreserved Mic-islets (C: 1000 capsules, n=5; D: 2000 capsules, n=4; E: 3000 capsules, n=2) with 15-deoxysperguarin. (E) At 41 days after implantation a mouse was killed for hematoxylin-eosin (H&E) and insulin staining. (F) The graft survival rate of recipients of Mic-islets and cryopreserved Mic-islets. Graft failure was defined as two consecutive plasma glucose determinations more than or equal to 200 mg/dL.

Mic-islets as a bioartificial pancreas in a xenotransplantation model. Recipients of 1000 and 2000 cryopreserved Mic-islets demonstrated 46 and 56 days of normoglycemia (Fig. 3C–D), indicating that cryopreserved Mic-islets could control the blood glucose levels of recipients and that the agarose hydrogel could protect xenogeneic islets from attack by the host's immune system. Although the function of Mic-islets was preserved during our cryopreservation process, the graft survival period of the cryopreserved 1000 Mic-islets (32.0±13.2 days) was shorter than that of noncryopreserved Mic-islets (53.2±16.7 days). During the cryopreservation process, islets were partially damaged and their insulin secreting ability was reduced. When 2000 cryopreserved Mic-islets were transplanted, the average period of the normoglycemia was prolonged to 46.3±13.3 days, which might be due to the reduction of the insulin secreting ability. In vitro and in vivo results indicated that the function and longevity of 2000 cryopreserved Mic-islets was approximately equivalent to that of 1000 noncryopreserved Mic-islets. The grafts retrieved

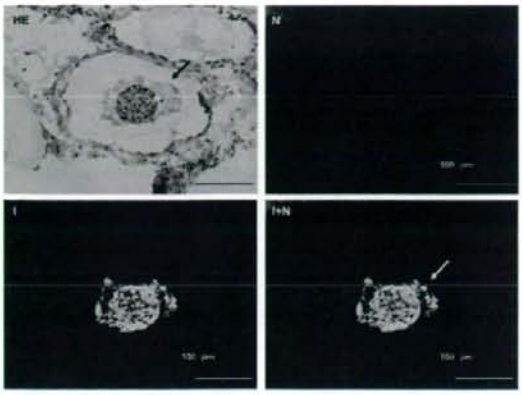

FIGURE 4. Histologic analysis of cryopreserved Mic-islets implanted in the intraperitoneal cavities of diabetic mice. These grafts were retrieved at 41 days posttransplantation. H&E, hematoxylin-eosin staining; N, Hoechst 33342 dye staining; I, insulin staining; I+N, merged images from Hoechst 33342 dye and insulin staining.

at 41 days posttransplantation were stained with an anti-insulin antibody, indicating that cryopreserved islets in agarose capsules had maintained strong insulin expression in vivo. Since the integrity of the agarose beads was well maintained after cryopreservation, the islets remained immunoisolated from tissues of recipients after transplantation (Fig. 4).

To our knowledge, this is the first report of successful cryopreservation of bioartificial pancreas and induction of long-term normoglycemia by transplantation of cryopreserved Mic-islets after a vitrification-warming process.

CONCLUSION

These data demonstrate that it is possible to cryopreserve agarose-encapsulated hamster islets using KYO-1 as a vitrification solution, although the insulin secreting ability of the islets was partially reduced. As a bioartificial pancreas, cryopreserved agarose-encapsulated islets successfully restored normoglycemia in diabetic mice for long periods of time.

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Islet encapsulation with living cells for improvement of biocompatibility

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ABSTRACT

Bioartificial pancreas, microencapsulation of islets of Langerhans (islets) within devices has been studied as a safe and simple technique for islet transplantation without the need for immuno-suppressive therapy. Various types of bioartificial pancreas have been proposed and developed such as microcapsule, macrocapsule and diffusion chamber types. However, these materials comprising a bioartificial pancreas are not completely inert and may induce foreign body and inflammatory reactions. The residual materials would be a problem in human body. Here we propose an alternative method for microencapsulation of islets with a layer of living cells. We immobilized HEK293 cells (human endoderm kidney cell line) to the islet surface using amphiphilic poly(ethylene glycol)-conjugated phospholid derivative and biotin/streptavidin reaction and encapsulated islets with a cell layer by culture. No necrosis of islet cells at the center was seen after microencapsulation with a layer of living cells. Insulin secretion ability by glucose stimulation was well maintained on these cell-encapsulated islets.

1. Introduction

Transplantation of islets of Langerhans (islets) has been proposed as a safe and effective method for treating patients with insulin-dependent diabetes mellitus (type I diabetes) although it was still an experimental procedure. In fact, the success achieved with the Edmonton protocol has established clinical islet transplantation as analternative to pancreas transplantation [1]. However, some improvements in islet transplantation are needed, such as increasing islet isolation efficiency, improving islets preservation, increasing the efficacy of immuno-suppressive drug dosage protocols, and reducing islet loss in the early phase following transplantation. A sufficient amount of islets for a recipient is isolated from a few donors. To overcome these issues, transplantation with islets enclosed in a semi-permeable membrane (i.e., a bioartificial pancreas) has been studied. Because islets are isolated from the host immune system by a semi-permeable membrane, they can survive and thus control glucose metabolism for a long period of time. Various types of bioartificial pancreas have been proposed and developed including islets micro-encapsulated within an alginate/poly(L-lysine) polyion complex membrane [2] or agarose hydrogel [3–6] or macroencapsulated by porous membranes [7–9]. Our group has developed a microcapsule type of bioartificial pancreas using agarose hydrogel and demonstrated the efficacy in diabetic animals. Recently we also have originally developed a new bioartificial pancreas with fibrinolytic property for transplantation into the liver through portal vein [10–13].

However, the materials comprising a bioartificial pancreas are not completely inert and might induce foreign body and inflammatory reactions. The consecutive fibrous tissue overgrowth diminishes the diffusion properties of nutrients, waste products, and oxygen as well as hormones such as insulin. The residual materials would be a problem because islet transplantation must be repeated when the blood glucose level is not controllable by residual grafts. Therefore, when we consider the use of a bioartificial pancreas in the clinical setting, it is necessary to suggest a new concept.

Here we propose a new method for the microencapsulation of islets with a layer of living cells using amphiphilic polymers, which would create a novel bioartificial pancreas. It is expected that a cell layer formed on the islet surface would be an immunoisolation membrane. When using living cells derived from a recipient for islet encapsulation, it should be possible to improve the biocompatibility of islets after transplantation. In this study, a layer of living HEK293 cells was formed on the surface of islets using the biotin–PEG–lipid and biotin/streptavidin reaction shown in Scheme 1.

2. Materials and methods

2.1. Materials

α-*N*-Hydroxysuccinimidyl-ω-*tert*-butoxycarbonyl poly(ethylene glycol) (NHS–PEG–*Boc*, MW: 5000) was purchased from Nektar Therapeutics (San Carlos, CA).

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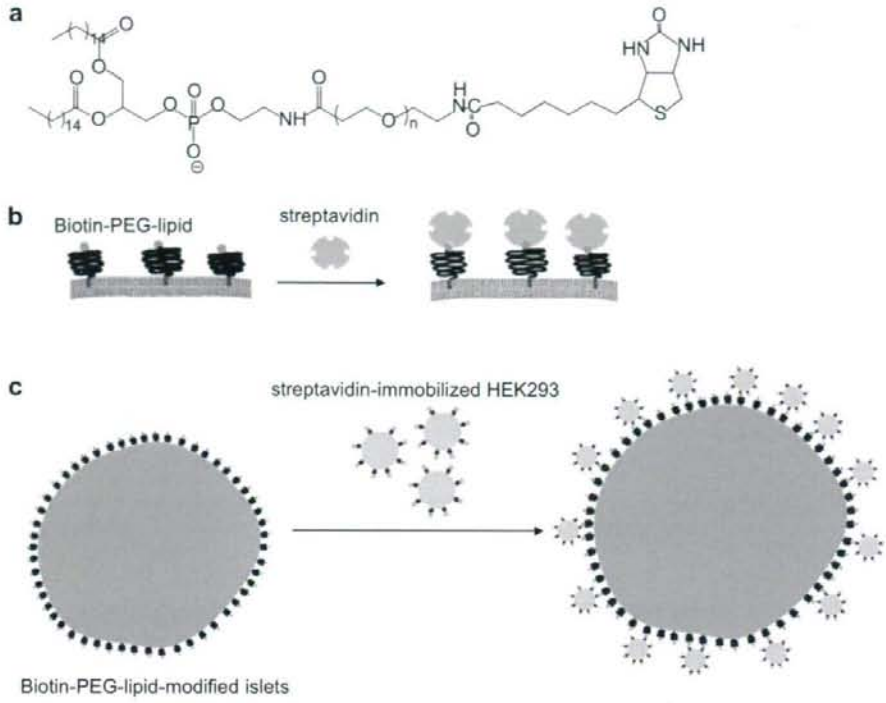

Scheme 1. (a) Chemical structure of biotin–PEG-conjugated DPPE (biotin–PEG–lipid). (b) Schematic illustration of the interaction between streptavidin and biotin–PEG–lipid at the lipid bilayer cell membrane. Biotin–PEG–lipid has hydrophobic acyl chains and is incorporated into the cell surface by anchoring into the lipid bilayer. Streptavidin was immobilized on the cell surface by anchoring to biotin–PEG–lipid. (c) Scheme for the immobilization of streptavidin-immobilized HEK293 cells on the surface of biotin–PEG–lipid-modified islets. After mixing streptavidin-immobilized HEK293 cells and biotin–PEG–lipid-modified islets, they were cultured in medium at 37 °C on a culture dish. During culture, HEK293 cells were spread and grown on the cell surface to cover the whole surface.

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; and streptavidin from *Streptomyces avidin* were purchased from Nacalai Tesque (Kyoto, Japan). Fluorescein isothiocyanate (FITC) and Hoechst 33342 were purchased from Dojindo Laboratories (Kumamoto, Japan). FITC-streptavidin was purchased from Zymed Laboratories (South San Francisco, CA). CellTracker®, the green fluorescent probe for the cell; Alexa 488-labeled goat anti-guinea pig IgG; minimum essential medium (MEM); HEPES buffer solution; Hanks' balanced salt solution; and Medium 199 were purchased from Invitrogen (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). The enzyme-linked immunosorbent assay (ELISA) kits for the insulin assay were purchased from Shibayagi Co., Ltd. (Gunma, Japan). Tissue-Tek was purchased from Sakura Fine Technical Co., Ltd. (Tokyo, Japan). Goat normal serum and polyclonal guinea pig anti-insulin were purchased from Dako (Glostrup, Denmark). The 10% formalin solution and Triton X-100 were purchased from Wako Pure Chemical (Osaka, Japan).

2.2. Synthesis of biotinylated PEG-conjugated DPPE

The NH_2–PEG–lipid was synthesized from DPPE and NHS–PEG–Boc, which carries an activated ester (NHS) that reacts with an amino group on DPPE, and a protective group (*Boc*) for the amino group, as reported previously [10]. Briefly, NHS–PEG–*Boc* (175 mg) and DPPE (21 mg) were dissolved in 5 mL 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 the *Boc* groups. The crude product was purified by precipitation with diethyl ether. After chloroform extraction 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 in 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 DMF evaporated *in vacuo*. Chloroform was then 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).

2.3. Encapsulation of islets with HEK293 cells

HEK293 (human endoderm kidney cell line) cells were obtained from the Health Science Research Resources Bank (Tokyo, Japan). The HEK293 cells were maintained in MEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin at 37 °C under 5% CO_2. The cells were collected by centrifugation (180 *g*, 5 min, RT) after treatment with trypsin. The cells were suspended in Hanks' balanced salt solution and then the medium exchanged. A cell pellet (4×10^6 cells) was obtained after centrifugation. After the addition of biotin–PEG–lipid solution (200 μL, 500 μg/mL) to the cell suspension, the suspension was incubated for 30 min with gentle agitation at RT. The cells were then suspended in 10 mL Hanks' balanced salt solution and collected by centrifugation (180 *g*, 5 min, 25 °C, twice) to obtain biotin–PEG–lipid-modified cells. Then, streptavidin (100 μL, 100 μg/mL) was added to the cell suspension and the suspension incubated for 30 min with gentle agitation at 4 °C. The cells were then suspended in 10 mL Hanks' balanced salt solution and collected by centrifugation (180 *g*, 5 min, 4 °C, twice) to obtain streptavidin-immobilized cells.

Islets were isolated from the pancreas of female Syrian hamsters (7–8 weeks old, Japan SLC, Inc., Shizuoka, Japan) using the collagenase digestion method. The islets were cultured for 7 days after isolation to remove or sediment cells damaged during the isolation procedure. The islets were maintained in culture medium (Medium 199 with 10% FBS, 8.8 mM HEPES buffer, 100 units/mL penicillin, 100 μg/mL streptomycin, and 8.8 U/mL heparin). A biotin–PEG–lipid solution was added to the islets suspended in serum-free MEM (200 islets, 500 μg/mL biotin–PEG–lipid, 100 μL of MEM), and the mixture was incubated at RT for 1.5 h. After washing three times with serum-free MEM, biotin–PEG–lipid-modified islets were obtained.

Finally, streptavidin-immobilized cells (4×10^6) and biotin–PEG–lipid-modified islets (200 islets) were mixed in serum-free MEM (300 μL), and the mixture was

incubated for 60 min with gentle agitation at RT. The HEK293 cell-immobilized islets were collected by hand and cultured on a non-treated dish at 37 °C under 5% CO_2 in Medium 199 supplemented with 10% FBS.

For visualization by confocal laser-scanning microscopy (FLUOVIEW FV500, Olympus, Tokyo, Japan), FITC-streptavidin and FITC-labeled PEG–lipid were used in treated HEK293 cells and islets. After the collection of HEK293 cells by trypsinization and centrifugation, cells were incubated with CellTracker® solution (30 μM) for 30 min at 37 °C in Hanks' balanced salt solution. The labeled green cells were incubated in serum containing medium for 30 min at 37 °C. Before surface modification with biotin–PEG–lipid, the cells were washed with Hanks' balanced salt solution.

2.4. Histochemical analysis

The HEK293 cell-immobilized islets were washed with PBS. Then, 10% formalin solution was added and the samples incubated for 12 h at RT. The formalin solution was removed and the islets sequentially maintained in 3% and 10% sucrose in PBS for 12 h, followed by incubation in 20% sucrose in PBS for 24 h at RT. The islets were embedded in Tissue-Tek for freezing. The frozen specimens were sliced (6-μm thick) using a cryostat (CM 3050S IV, Leica, Solms, Germany). The sliced sections were permeabilized with 0.2% Triton X-100 in PBS at RT for 15 min. The samples were then treated with a 10% normal goat serum in PBS for 1 h to block the non-specific binding of antibodies. The samples were then treated with 1% polyclonal guinea pig anti-insulin in PBS containing 3% goat normal serum for 3.5 h at RT, and then washed with PBS. The samples were incubated with fluorescently labeled secondary antibody, 0.2% Alexa 488 Goat anti-guinea pig IgG in PBS containing 3% goat normal serum, at RT for 1.5 h. Cell nuclei were counterstained with Hoechst 33342. The localization of secondary antibodies and the Hoechst dye was analyzed by fluorescence microscopy (IX71, Olympus Optical Co., Ltd., Tokyo, Japan). The sliced sections were also stained with hematoxylin–eosin (HE) using a conventional staining method.

2.5. Insulin secretion from HEK293 cell-immobilized islets on glucose stimulation

Static insulin secretion tests were performed on HEK293 cell-immobilized islets (50 islets) after culturing HEK293 cell-immobilized islets for 3 days to evaluate their insulin-secreting ability in response to changes in glucose concentration. As a control experiment, this assay was performed on bare islets. HEK293 cell-immobilized 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 h at 37 °C for each concentration. The solutions were collected after each 1-h incubation. The insulin concentration in the solutions was determined by ELISA.

3. Results

3.1. Cell surface modification with PEG–lipid derivatives

A layer of living HEK293 cells was formed on the surface of islets using amphiphilic polymers, biotin–PEG–lipid and biotin/streptavidin reaction as shown in Scheme 1. We have studied the surface modification of living cells and islets with amphiphilic polymers such as PEG-conjugated phospholipid and poly(vinyl alcohol) carrying alkyl side chains for the improvement of graft survival in cell transplantation [10–14]. The method is promising because there were no damages to cell function after incorporation into the phospholipid bilayer membrane by hydrophobic interaction.

Using PEG–lipids carrying a biotin molecule at the end of the PEG chain (biotin–PEG–lipid, Scheme 1a), biotin molecules were introduced on the cell surface (Scheme 1b). Then, streptavidin was immobilized on the biotin–PEG–lipid-modified surface. Unoccupied sites on streptavidin were available for cell-immobilization because streptavidin has four binding sites for biotin. First, the surfaces of single HEK293 cells were treated with biotin–PEG–lipid. Then, after incubation in biotin–PEG–lipid dispersion, FITC-labeled streptavidin was added. Fig. 1a shows microscopic images of cells modified with FITC-labeled streptavidin/biotin–PEG–lipid obtained with a confocal laser-scanning microscope. Fluorescence from FITC-streptavidin was visible at the periphery of each cell, indicating the formation of an FITC-streptavidin and biotin–PEG–lipid complex on the cell membrane. No fluorescence was observed for cells not treated with biotin–PEG–lipid. Islets were also treated with a mixture of biotin–PEG–lipid and FITC-labeled PEG–lipid (Fig. 1b). We expect that the PEG–lipid derivatives behaved in a similar way because they are similar in chemical structure. Clear fluorescence was seen at the periphery of each islet. These observations indicate that biotin groups can be introduced onto the surface of islets using biotin–PEG–lipid without damaging islet morphology.

3.2. Islets' encapsulation with living HEK293 cells

Next, we immobilized HEK293 cells at the surface of islets by reacting streptavidin-immobilized HEK293 cells with biotin–PEG–lipid-modified islets (Fig. 1c). Prior to this step, the HEK293 cells had been fluorescently labeled with CellTracker®. Fluorescently labeled HEK293 cells were observed at the surface of islets, indicating that the HEK293 cells were immobilized on the islet surface by the biotin/streptavidin reaction. The HEK293 cell-immobilized islets were cultured in medium on a culture dish. The HEK293 cells were spread and gradually proliferated on the islet surface (Fig. 2). Immobilized HEK293 cells on the islet surface were identified as white cells (arrows in Fig. 2), which were apparently different from the islets. The HEK293 cells on the islet surface were spherical in shape just after immobilization. Then HEK293 cells were attached and spread on the surface after 1 day of culture. After 3 days in culture, the islet surface was covered with a layer of HEK293 cells, indicating that the HEK293 cells proliferated on the islet surface. The islets were completely encapsulated by a layer of HEK293 cells after 5 days in culture.

3.3. Histochemical analysis

The HEK293 cell-encapsulated islets were histochemically analyzed by HE and insulin staining (Fig. 3). In HE staining, a layer of HEK293 cells was observed around the islet surface after culturing for 3 and 5 days (Fig. 2). The nucleus of HEK293 cells was slightly larger than that of islets; therefore, single- and multi-layers of HEK293 cells were formed on the islet surface after culturing for 3 and 5 days, respectively. Islet cell necrosis at the center was not observed after encapsulation with HEK293 cells. Fig. 3b shows images of insulin-stained HEK293 cell-encapsulated islets. Islets stained with anti-insulin antibody (green) were covered with HEK293 cells. A layer of HEK293 cells stained for nuclei (Hoechst 33342) but not insulin. These results indicate that HEK293 cell-encapsulation did not impair the ability of islets to produce insulin, and that the HEK293 cell layer permits the diffusion of oxygen, nutrients, glucose, and insulin.

3.4. Insulin secretion by glucose stimulation

A glucose stimulation test was performed to examine the ability to control insulin release in response to changes in the glucose level (Fig. 4). When the glucose concentration was increased from 0.1 g/dL to 0.3 g/dL, two groups of islets increased insulin release from basal levels. Insulin release returned to basal levels when the islets were re-exposed to 0.1 g/dL glucose. These results indicate that HEK293 cell-encapsulation did not influence the islets' ability to regulate and release insulin, although a decrease in the amount of insulin secretion was seen. The decrease after glucose stimulation might be due to the low permeability of the HEK293 cell layer to insulin.

4. Discussion

Various types of bioartificial pancreas have been proposed and developed for treating type I diabetic patients such as islets microencapsulated within an alginate/poly(L-lysine) polyion complex membrane [2] or agarose hydrogel [3–6] or

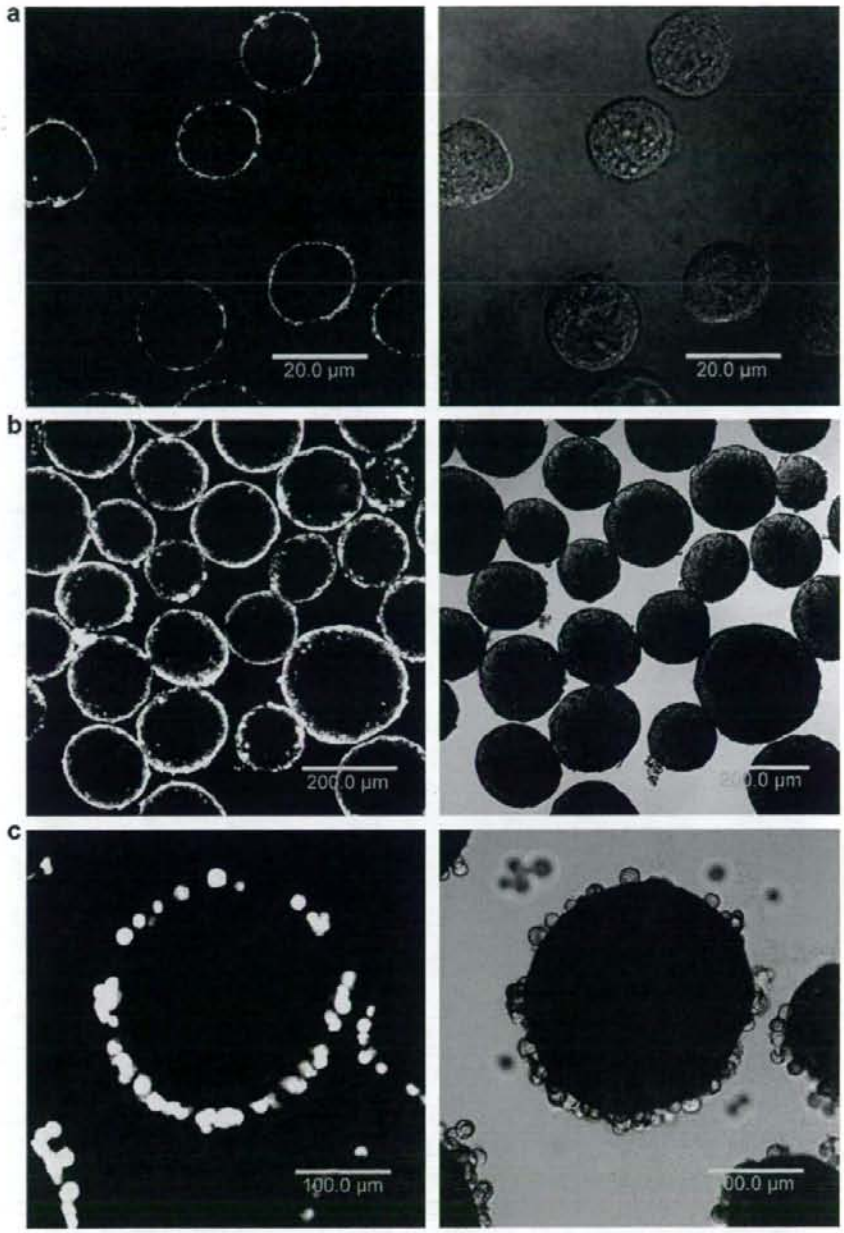

Fig. 1. Confocal laser-scanning and differential interference microscope images of surface-modified cells and islets. (a) HEK293 cells that were sequentially modified with biotin–PEG–lipid and FITC-labeled streptavidin. (b) Hamster islets modified with a mixture of biotin–PEG–lipid and FITC-labeled PEG–lipid. (c) Hamster islets modified with biotin–PEG–lipid and immobilized with streptavidin-immobilized HEK293 cells. The HEK293 cells were labeled with CellTracker®. Scale bars: 20 μm (a), 200 μm (b), and 100 μm (c).

macroencapsulated by porous membranes [7–9]. Most bioartificial pancreas are made of synthetic and natural polymers, and there are a few reports of cell-based bioartificial pancreas. For example, Pollok et al. first reported that it was possible to macroencapsulate islets from rats with porcine chondrocyte membrane [15]. Porcine chondrocytes were cultured to form a confluent monolayer and deposit matrix. After islets were attached to the poly(glycolic acid) polymer fibers, the hybrid was wrapped with chondrocyte membrane by hand. However, the total volume of the implant tended to increase after macroencapsulation of the islets with the

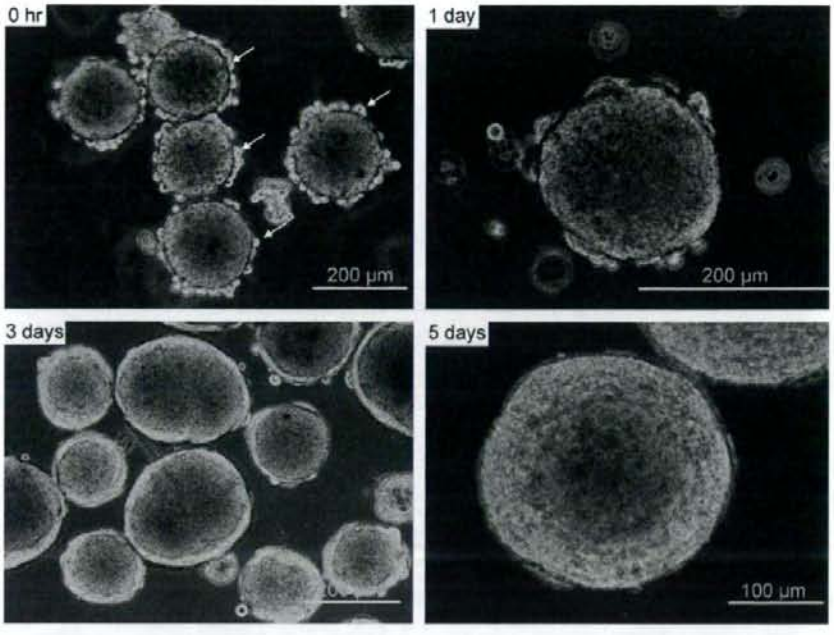

Fig. 2. Phase-contrast microscopy of HEK293 cell-immobilized islets in culture at 0, 1, 3, and 5 days. HEK293 cells were immobilized on the surface of the islets and cultured on a non-treated dish in Medium 199 at 37 °C. Arrows indicate immobilized HEK293 cells.

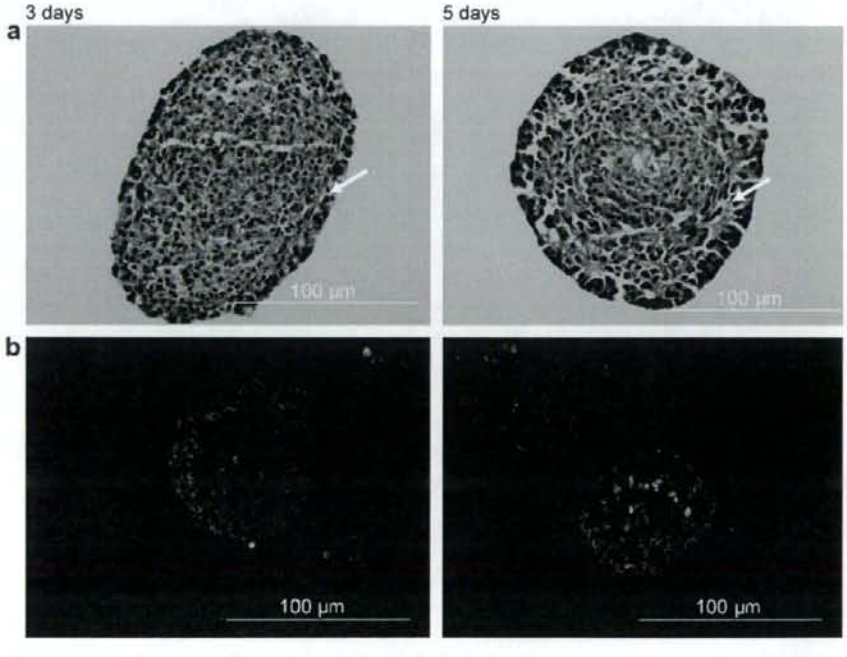

Fig. 3. Histochemical analysis of HEK293 cell-immobilized islets cultured for 3 and 5 days in medium. Frozen sections of HEK293 cell-immobilized islets were stained with (a) hematoxylin–eosin (HE) or (b) Alexa 488-labeled anti-insulin antibody and Hoechst 33342 dye for nuclear staining. The pictures are merged images from insulin and Hoechst 33342 staining. The arrows in (a) indicate a boundary between islet cells and HEK293 cells.

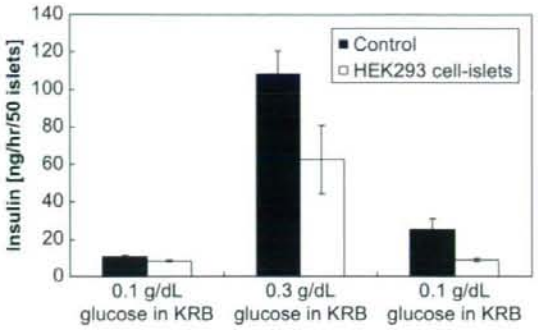

Fig. 4. Glucose stimulation test of HEK293 cell-immobilized islets cultured for 3 days in medium. As a control experiment, the assay was performed on bare islets. The amount of insulin secreted from the islets in response to glucose concentration changes (0.1, 0.3, 0.1 g/dL) was determined by ELISA. Results are expressed as mean ± SD for $n = 3$. KRB = Krebs–Ringer buffer.

membrane, which limited the transplantation sites. Moreover, fusion between islets inside the chondrocyte membrane causes the necrosis of islet cells at the center. Lee et al. also demonstrated macroencapsulation of islets with chondrocyte membrane using the cell sheet engineering technique [16]. However, the issues have not yet been solved. Therefore, some aspects of the cell-based encapsulation methods should be improved.

To overcome these problems, we attempted to microencapsulate islets with a cell layer membrane. To our knowledge, there have been no previous reports of microencapsulating islets with living cells. Here we used amphiphilic PEG–lipid and biotin/streptavidin reaction to immobilize HEK293 cells on the surface of islets. The surface of the islets was completely covered with a cell layer after 3–5 days in culture without central necrosis of the islet cells (Figs. 2 and 3). Insulin secretion upon glucose stimulation was well maintained on HEK293 cell-encapsulated islets, though reduced compared to bare islets (Fig. 4). It was thought that the permeability of the HEK293 cell layer to insulin was slightly low. Thus, we succeeded in the first microencapsulation of islets with a cell layer, although HEK293 cells are a cell line. Using our technique, it will be possible to microencapsulate islets with cells derived from type I diabetic recipients. We expect that this novel bioartificial pancreas will have high biocompatibility because inflammatory reactions and immunorejection should be suppressed after transplantation. However, many important issues still remain, as discussed above, which mainly can be addressed by *in vivo* studies. The results will be reported in the near future.

5. Conclusions

We succeeded in immobilization of living cells to the islet surface using amphiphilic PEG–lipid derivative and biotin/streptavidin reaction, and microencapsulation of islet surface with a layer of cells without central necrosis of islet cells. Insulin secretion ability by glucose stimulation was well maintained on these cell-encapsulated islets.

Acknowledgements

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Appendix

Figures with essential colour discrimination. The Scheme and the majority of the figures in this article are difficult to interpret in black and white. The full colour images can be found in the online version, at doi:10.1016/j.biomaterials.2009.01.036.

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