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

Agudelo G. Carlos,[1,3] *Yuji Teramura,*[2] *and Hiroo Iwata*[1,4]

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

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

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

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

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

(*Transplantation* 2009;87: 29–34)

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

This work was supported by the Japanese Society for the Promotion of Science (JSPS) for the Postdoctoral Fellowship for Foreign Researchers (Grant-in-Aid for Scientific Research [Tokubetsu Kenkyuin Shorei-hi]), R&D Projects for Economic Reinvigoration (Leading Projects), and Grant-in-Aid for Scientific Research (A) (No. 18200034) from the Japanese Ministry of Education, Culture, Sports, Science, and Technology (MEXT).

[1] Department of Reparative Materials, Institute for Frontier Medical Sciences, Kyoto University, Sakyo-ku, Kyoto, Japan.

[2] Department of Polymer Chemistry, Graduate School of Engineering, Kyoto University, Sakyo-ku, Kyoto, Japan.

[3] Currently, Department of Biomedical Engineering, National Cardiovascular Center Research Institute, Fujishirodai, Suita, Osaka, Japan.

[4] Address correspondence to: Hiroo Iwata, Ph.D., Institute for Frontier Medical Sciences, Kyoto University 53 Kawara-cho, Shogin, Sakyo-ku.

E-mail: iwata@frontier.kyoto-u.ac.jp

Received 4 August 2008

Accepted 10 September 2008.

Copyright © 2009 by Lippincott Williams & Wilkins

ISSN 0041-1337/09/8701-29

DOI: 10.1097/TP.0b013e318191b24b

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

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

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

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

MATERIALS AND METHODS

Microencapsulation of Islets in Agarose Hydrogel

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

Cryopreservation of Mic-Islets in Vitrification Solution

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

Warming of Cryopreserved Mic-Islets

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

Evaluation of Cryopreserved Mic-Islets "in vitro"

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

Evaluation of Cryopreserved Mic-Islets "in vivo"

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

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

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

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

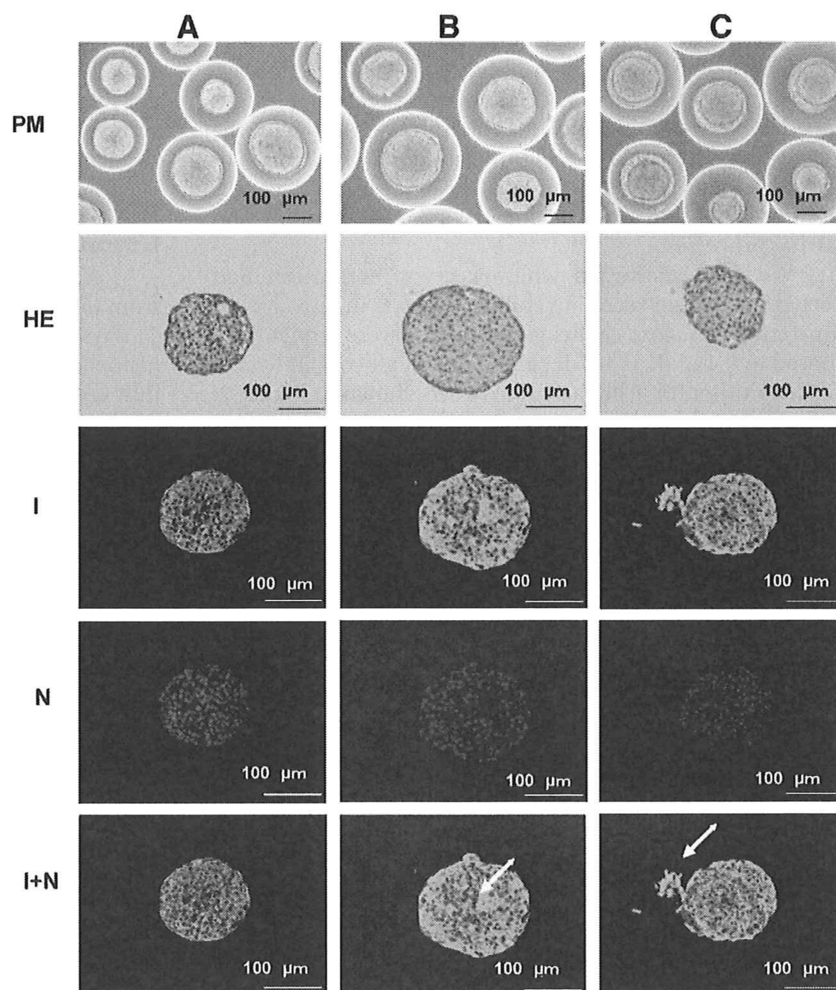


FIGURE 1. Morphology and histology of Mic-islets after cryopreservation and thawing and culture for 1 day or 1 week. (A) Mic-islets cultured for 1 week without cryopreservation as a control. Cryopreserved Mic-islets cultured for (B) 1 day and (C) 1 week after thawing. The morphology of the Mic-islets 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.

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

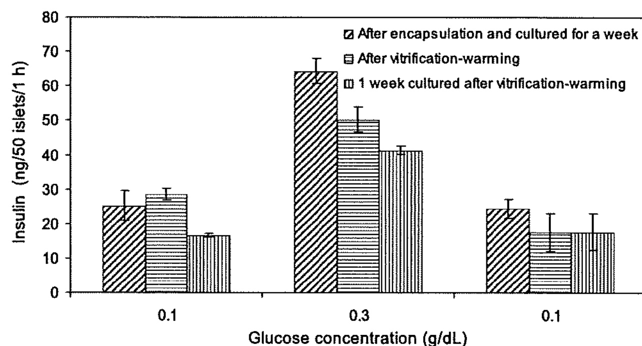


FIGURE 2. Amounts of insulin secreted by Mic-islets and cryopreserved Mic-islets in response to glucose stimulation. Cryopreserved Mic-islets were thawed, warmed, and cultured for 1 day (n=3) and 1 week (n=3). Mic-islets cultured for 1 week without cryopreservation were used as controls (n=3). Data are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

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

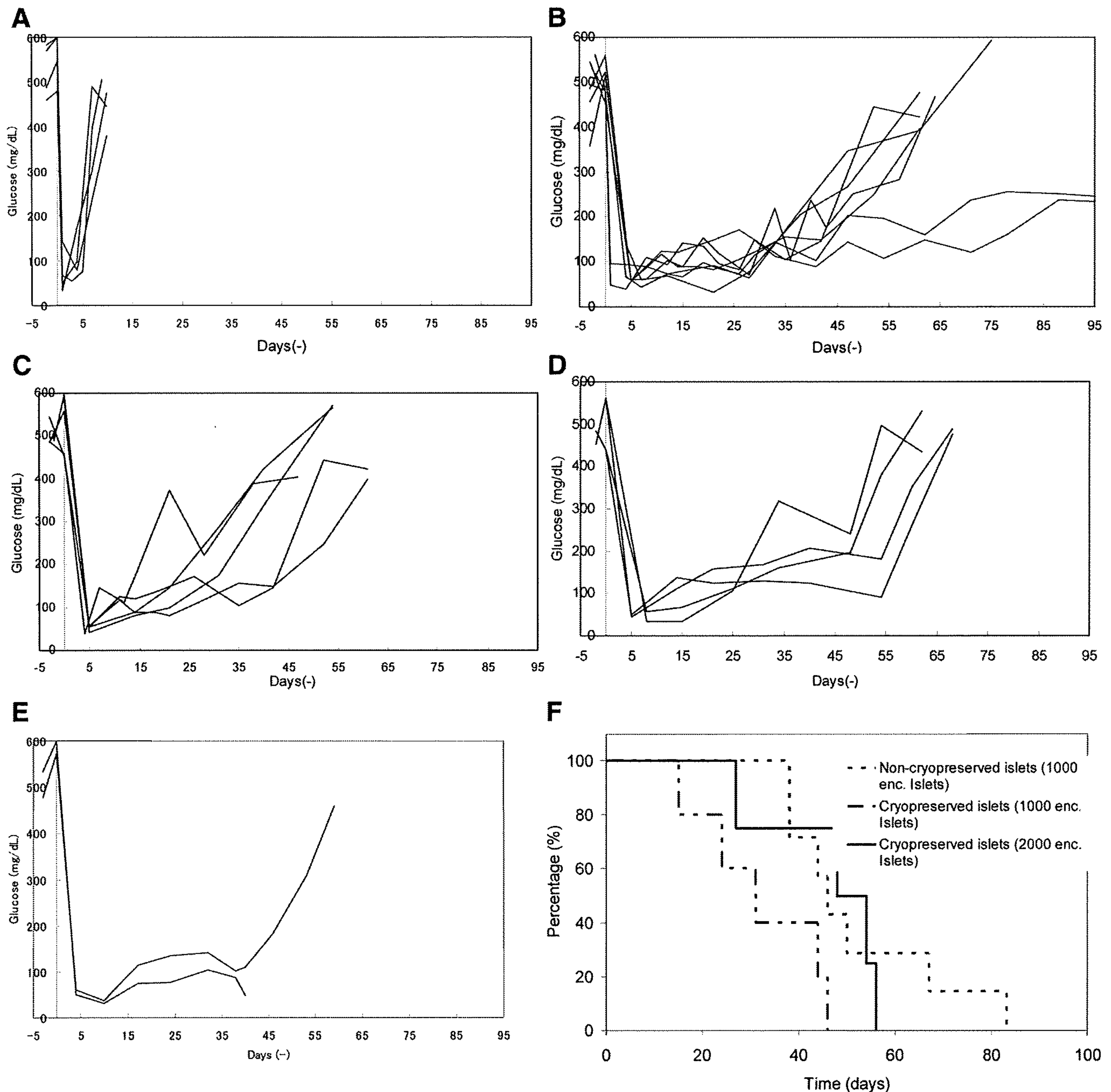


FIGURE 3. Changes in nonfasting blood glucose levels of streptozotocin-induced diabetic Balb/c mice after intraperitoneal implantation of (A) bare islets (1000 islets) without 15-deoxysperguarin, (B) Mic-islets (1000 capsules, n=7) and cryopreserved Mic-islets (C: 1000 capsules, n=5; D: 2000 capsules, 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

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

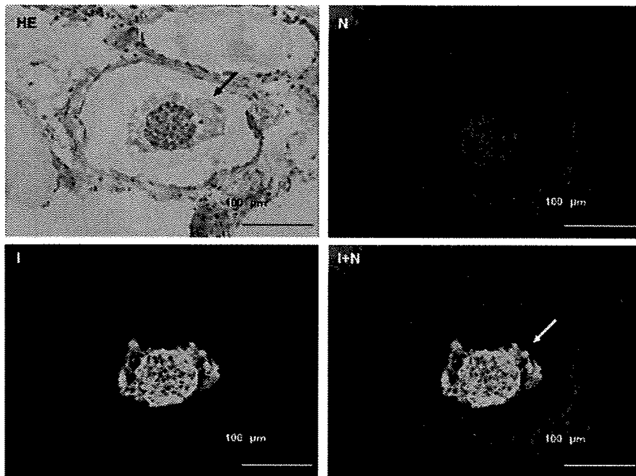


FIGURE 4. Histologic analysis of cryopreserved Mic-islets implanted in the intraperitoneal cavities of diabetic mice. These grafts were retrieved at 41 days posttransplantation. H&E, hematoxylin-eosin staining; N, Hoechst 33342 dye staining; I, insulin staining; I+N, merged images from Hoechst 33342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

ACKNOWLEDGMENTS

The authors thank Takahiko Totani for animal care assistance. Agudelo C. thanks the Japanese Society for the Promotion of Science (JSPS) for the Postdoctoral Fellowship for Foreign Researchers.

REFERENCES

1. Shapiro AM, Lakey JR, Ryan EA, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N. *Engl J Med* 2000; 343: 230.
2. Lim F, Sun AM. Microencapsulated islets as bioartificial endocrine pancreas. *Science* 1980; 210: 908.
3. Lanza RP, Sullivan SJ, Chick W. Islets transplantation with immunoisolation. *Diabetes* 1992; 41: 1503.
4. Omer A, Duvivier-Kali V, Fernandes J, et al. Long-term normoglycemia in rats receiving transplants with encapsulated islets. *Transplantation* 2005; 79: 52.
5. Lee DY, Nam JH, Byun Y. Functional and histological evaluation of transplanted pancreatic islets immunoprotected by PEGylation and cyclosporine for 1 year. *Biomaterials* 2007; 28: 1957.
6. Iwata H, Takagi T, Amemiya H, et al. Agarose for a bioartificial pancreas. *J Biomed Mater Res* 1992; 26: 967.
7. Iwata H, Kobayashi K, Takagi T, et al. Feasibility of agarose microbeads with xenogeneic islets as a bioartificial pancreas. *J Biomed Mater Res* 1994; 28: 1003.
8. Kobayashi T, Aomatsu Y, Iwata H. Indefinite islet protection from autoimmune destruction in nonobese diabetic mice by agarose microencapsulation without immunosuppression. *Transplantation* 2003; 75: 619.
9. Yang H, Iwata H, Shimizu H, et al. Comparative studies of in vitro and in vivo function of three different shaped bioartificial pancreases made of agarose hydrogel. Biomaterials. 1994;15: 113.
10. Rajotte RV, Stewart HL, Voss WAG, et al. Viability studies on frozen-thawed rat islets of Langerhans. *Cryobiology* 1977; 14: 116.
11. Rajotte RV, Warnock GL, Kneteman NN. Cryopreservation of insulin-producing tissue in rats and dogs. *World J Surg* 1984; 8: 179.
12. Rajotte RV, Warnock GL, Kneteman NM, et al. Optimizing cryopreservation of isolated islets. *Transplantation Proc* 1989; 21: 2638.
13. von Mach MA, Schlosser J, Weiland M, et al. Size of pancreatic islets of Langerhans: A key parameter for viability after cryopreservation. *Acta Diabetol* 2003; 40: 123.
14. Matsumoto S, Matsusita M, Morita T, et al. Effects of synthetic antifreeze glycoprotein analogue on islet cell survival and function during cryopreservation. *Cryobiology* 2006; 52: 90.
15. Agudelo CA, Iwata H. The development of alternative vitrification solutions for microencapsulated islets. *Biomaterials* 2008; 29: 1167.
16. Iwata H, Takagi T, Amemiya H. Marked prolongation of islets xenograft survival (hamster to mouse) by microencapsulation and administration of 15-deoxyspergualin. *Transplant Proc* 1992; 24: 1517.
17. Lacy PE, Kostianovsky M. A method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes* 1967; 16: 35.
18. Song YC, Khirabadi BS, Lightfoot F, et al. Vitreous cryopreservation maintains the function of vascular grafts. *Nat Biotech* 2000; 18: 296.

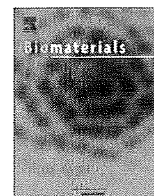
Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

Biomaterials 30 (2009) 2270–2275

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials



Islet encapsulation with living cells for improvement of biocompatibility

Yuji Teramura [a,b], Hiroo Iwata [c,*]

[a] Department of Nano-Medicine Merger Education Unit, Graduate School of Engineering, Kyoto University, Kyoto 606-8507, Japan
[b] Department of Polymer Chemistry, Graduate School of Engineering, Kyoto University, Kyoto 606-8507, Japan
[c] Institute for Frontier Medical Sciences, Kyoto University, 53 Kawara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

ARTICLE INFO

Article history:
Received 8 January 2009
Accepted 19 January 2009
Available online 6 February 2009

Keywords:
Bioartificial pancreas
Islet
Cell-encapsulation
Microencapsulation
Poly(ethylene glycol)–lipid (PEG–lipid)

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.

© 2009 Elsevier Ltd. All rights reserved.

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.

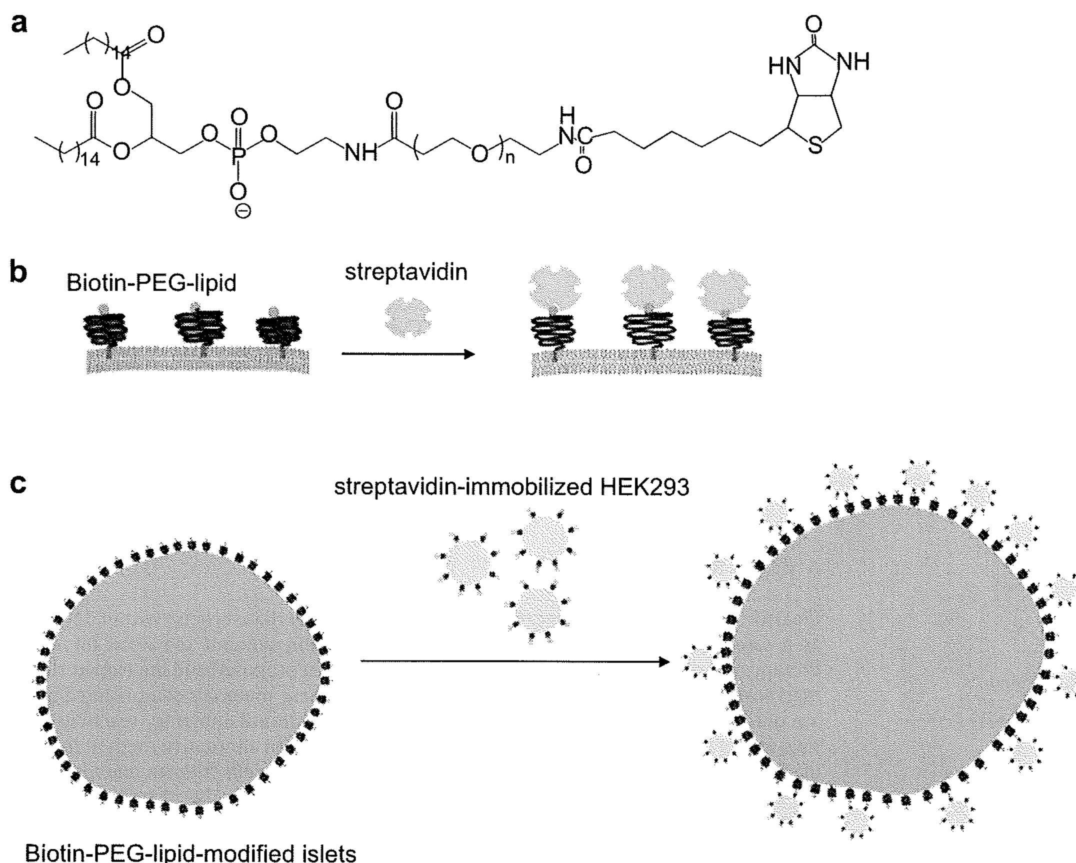
2. Materials and methods

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).

* Corresponding author. Tel./fax: +81 75 751 4119.
E-mail address: iwata@frontier.kyoto-u.ac.jp (H. Iwata).

0142-9612/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2009.01.036



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 ^1H 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

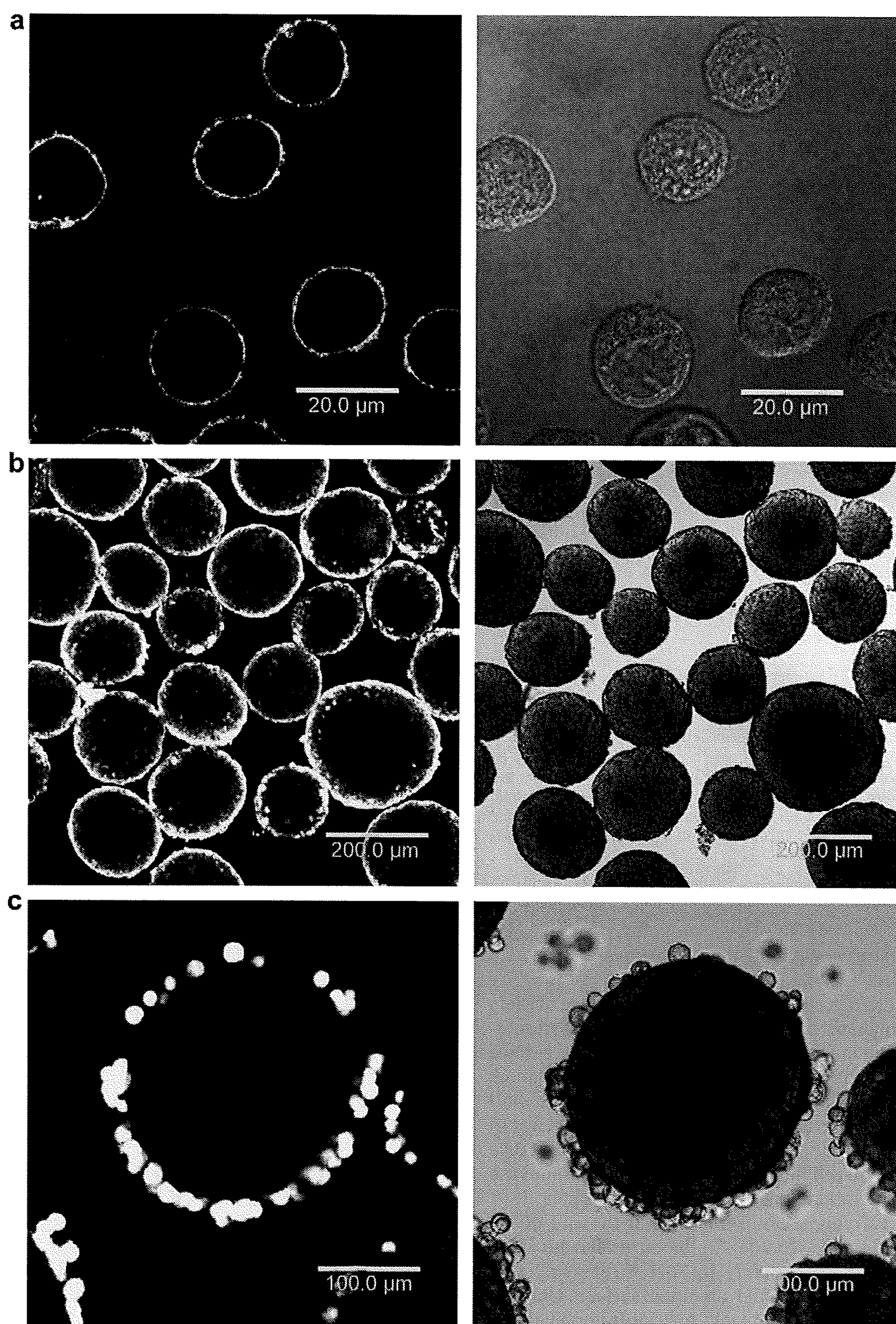


Fig. 1. Confocal laser-scanning and differential interference microscope images of surface-modified cells and islets. (a) HEK293 cells that were sequentially modified with biotin–PEG–lipid and FITC-labeled streptavidin. (b) Hamster islets modified with a mixture of biotin–PEG–lipid and FITC-labeled PEG-lipid. (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

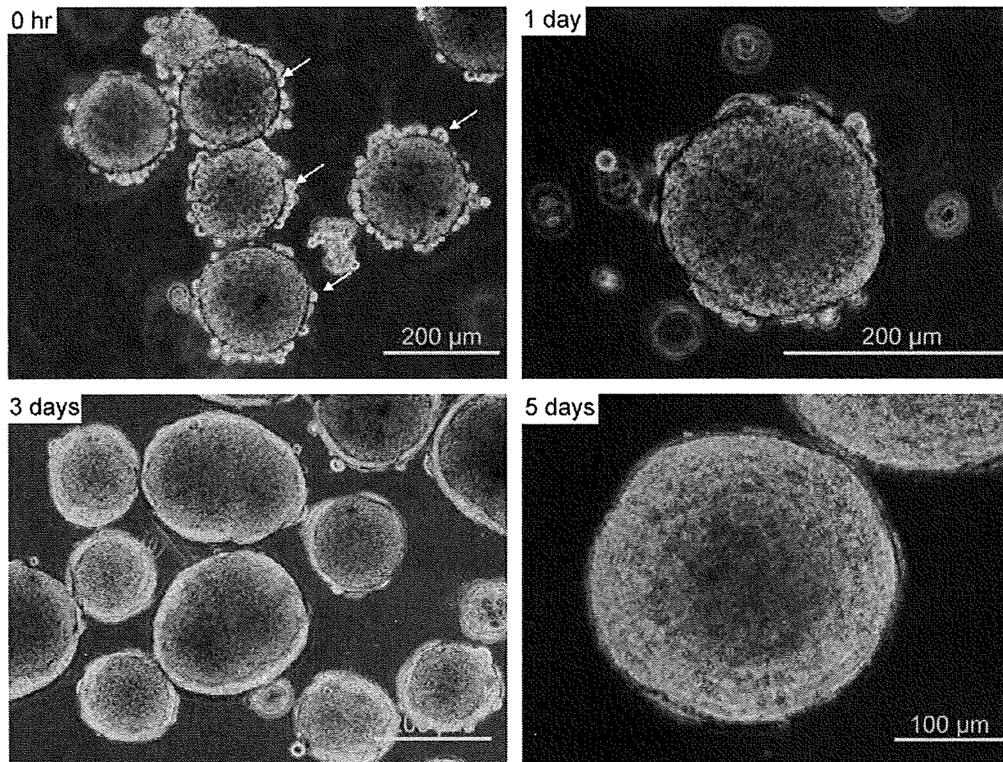


Fig. 2. Phase-contrast microscopy of HEK293 cell-immobilized islets in culture at 0, 1, 3, and 5 days. HEK293 cells were immobilized on the surface of the islets and cultured on a non-treated dish in Medium 199 at 37 °C. Arrows indicate immobilized HEK293 cells.

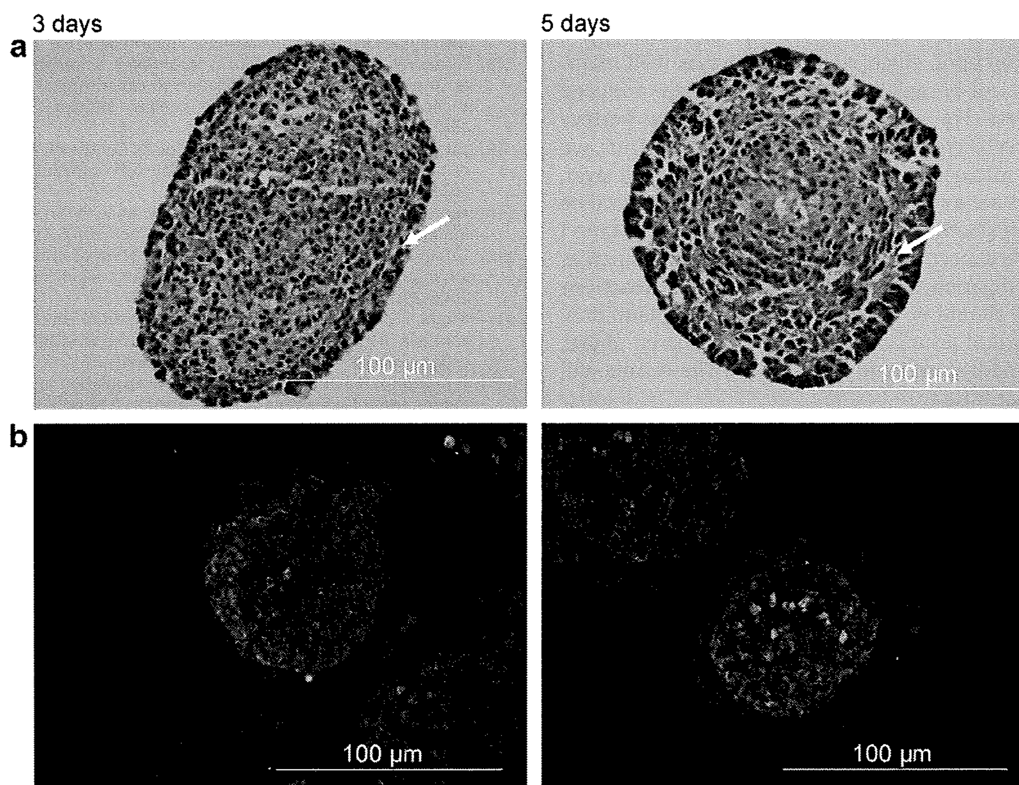


Fig. 3. Histochemical analysis of HEK293 cell-immobilized islets cultured for 3 and 5 days in medium. Frozen sections of HEK293 cell-immobilized islets were stained with (a) hematoxylin–eosin (HE) or (b) Alexa 488-labeled anti-insulin antibody and Hoechst 33342 dye for nuclear staining. The pictures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

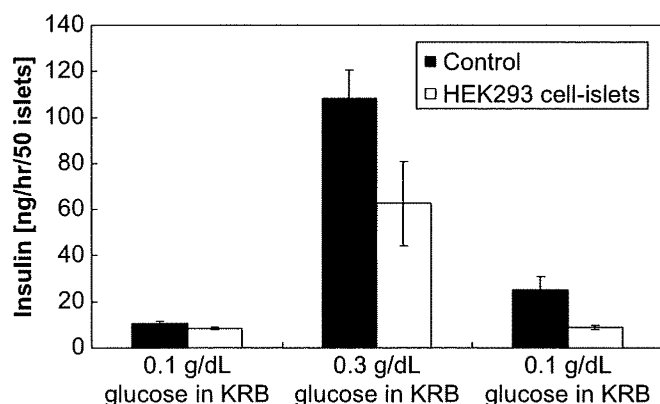


Fig. 4. Glucose stimulation test of HEK293 cell-immobilized islets cultured for 3 days in medium. As a control experiment, the assay was performed on bare islets. The amount of insulin secreted from the islets in response to glucose concentration changes (0.1, 0.3, 0.1 g/dL) was determined by ELISA. 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

This study was supported in part by CREST and JST, and by a Grant-in-Aid for Scientific Research (A) (No. 18200034) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan and by the Ministry of Health, Labour, and Welfare of Japan (H20-007).

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.

References

[1] Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 2000;343:230–8.
[2] Lin F, Sun AM. Microencapsulated islets as bioartificial endocrine pancreas. Science 1980;210:908–10.
[3] Iwata H, Takagi T, Shimizu H, Yamashita K, Kobayashi K, Akutsu T. Agarose for bioartificial pancreas. J Biomed Mater Res 1992;26:967–77.
[4] Iwata H, Kobayashi K, Takagi T, Oka T, Yang H, Amemiya H, et al. Feasibility of agarose microbeads with xenogeneic islets as a bioartificial pancreas. J Biomed Mater Res 1994;28:1003–11.
[5] Kobayashi T, Aomatsu Y, Iwata H. Indefinite islet protection from autoimmune destruction in nonobese diabetic mice by agarose microencapsulation without immunosupression. Transplantation 2003;75:619–25.
[6] Agudelo CA, Teramura Y, Iwata H. Cryopreserved agarose-encapsulated islets as bioartificial pancreas: a feasibility study. Transplantation 2009;87:29–34.
[7] Lanza RP, Sullivan SJ, Chick WL. Treatment of severely diabetic pancreatectomized dogs using a diffusion-based hybrid pancreas. Diabetes 1992;41:886–9.
[8] Altman JJ, Houlbert D, Callard P, McMillan P, Solomon BA, Rosen J, et al. Long-term plasma glucose normalization in experimental diabetic rats with macroencapsulated implants of benign human insulinomas. Diabetes 1986;35:625–33.
[9] Lacy PE, Hegre OD, Gerasimidi-Vazeou A, Gentile FT, Dionne KE. Maintenance of normoglycemia in diabetic mice by subcutaneous xenografts of encapsulated islets. Science 1991;254:1782–4.
[10] Teramura Y, Iwata H. Islets surface modification prevents blood-mediated inflammatory responses. Bioconjugate Chem 2008;19:1389–95.
[11] Totani T, Teramura Y, Iwata H. Immobilization of urokinase to islet surface by amphiphilic poly (vinyl alcohol) carrying alkyl side chains. Biomaterials 2008;29:2878–83.
[12] Miura S, Teramura Y, Iwata H. Encapsulation of islets with ultra-thin polyion complex membrane through poly(ethylene glycol)-phospholipids anchored to cell membrane. Biomaterials 2006;27:5828–35.
[13] Teramura Y, Kaneda Y, Iwata H. Islet-encapsulation in ultra-thin layer-by-layer membranes of poly(vinyl alcohol) anchored to poly(ethylene glycol)-lipids in the cell membrane. Biomaterials 2007;28:4818–25.
[14] Teramura Y, Kaneda Y, Totani T, Iwata H. Behavior of synthetic polymers immobilized on cell membrane. Biomaterials 2008;29:1345–55.
[15] Lee JI, Nishimura R, Sakai H, Sasaki N, Kenmochi T. A newly developed immunoisolated bioartificial pancreas with cell sheet engineering. Cell Transplant 2008;17:51–9.
[16] Pollok JM, Lorenzen M, Kölln PA, Török E, Kaufmann PM, Kluth D, et al. In vitro function of islets of Langerhans encapsulated with a membrane of porcine chondrocytes for immunoisolation. Dig Surg 2001;18:204–10.

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

This study was supported by CREST and JST (in part); the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, grant-in-aid for Scientific Research (A) nos. 18200034 and 21240051; and the Ministry of Health, Labour and Welfare, Japan, grant H20-007.
The authors declare no conflict of interest.
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.

[1] Department of Nano-Medicine Merger Education Unit, Graduate School of Engineering, Kyoto University, Kyoto, Japan. Currently, Radioisotope Research (RI) Center, Kyoto University, Kyoto, Japan.
[2] Department of Polymer Chemistry, Graduate School of Engineering, Kyoto University, Kyoto, Japan. Currently, Radioisotope Research (RI) Center, Kyoto University, Kyoto, Japan.
[3] Department of Reparative Materials, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan.
[4] Address correspondence to: Yuji Teramura, Ph.D., Radioscope Research (RI) Center, Kyoto University, Yoshida-Konoe-Cho, Sakyo-Ku, Kyoto, 606-8501, Japan.
E-mail: teramura@frontier.kyoto-u.ac.jp

Received 1 December 2008. Revision requested 25 May 2009.
Accepted 3 June 2009.
Copyright © 2009 by Lippincott Williams & Wilkins
ISSN 0041-1337/09/8805-624
DOI: 10.1097/TP.0b013e3181b230ac

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

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-

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

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

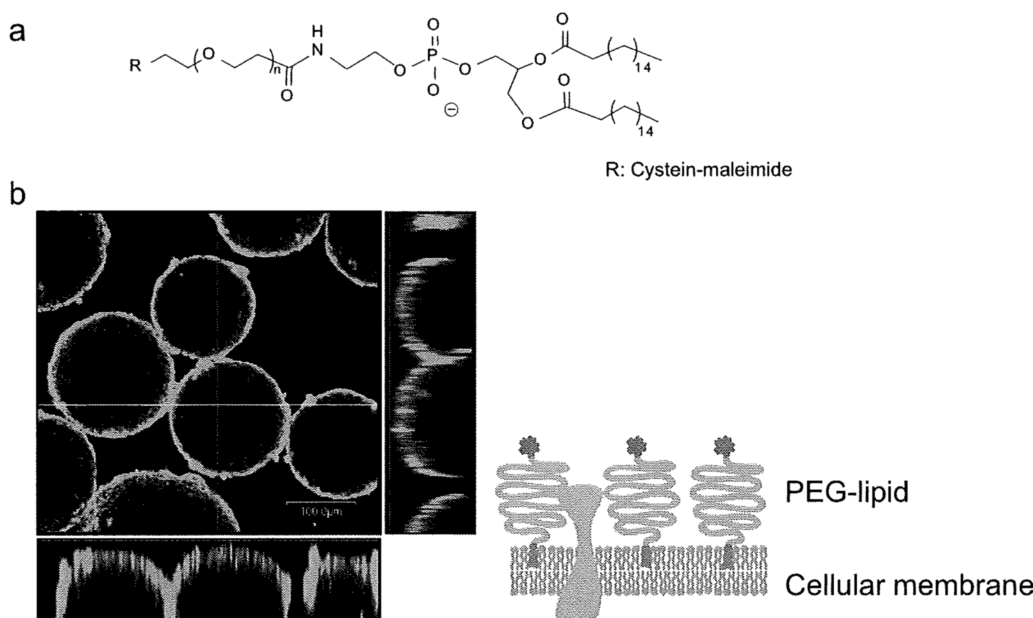


FIGURE 1. (a) Chemical structure of poly(ethylene glycol) (PEG)-conjugated dipalmitoyl-*sn*-glycerol-3-phosphatidylethanolamine (PEG-lipid). (b) Confocal laser scanning images of fluorescein isothiocyanate-PEG-lipid-modified islets from hamsters. PEG-lipid can be introduced on the surface of islets by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

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

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.

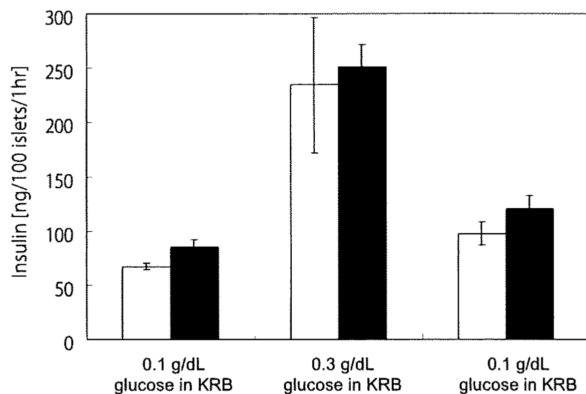


FIGURE 2. Assessment of insulin release in glucose stimulated islets. The amounts of insulin secreted from poly(ethylene glycol) islets (*black bars*) and control bare islets (*white bars*) in response to glucose concentration changes (0.1, 0.3, and 0.1 g/dL) were determined by ELISA. Results are expressed 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

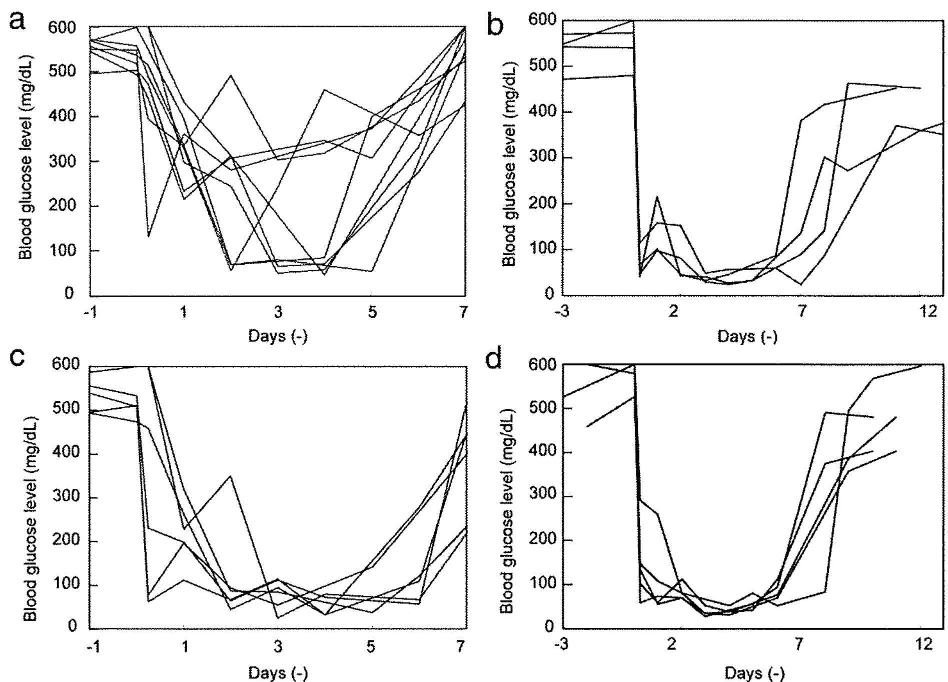


FIGURE 3. Changes in nonfasting blood glucose levels of streptozotocin-induced diabetic Balb/c mice after intraportal transplantation of (a) 500 control islets (n=9), (b) 1000 control islets (n=4), (c) 500 poly(ethylene glycol) islets (n=6), or (d) 1000 poly(ethylene glycol) islets (n=5). Graft failure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

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

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.

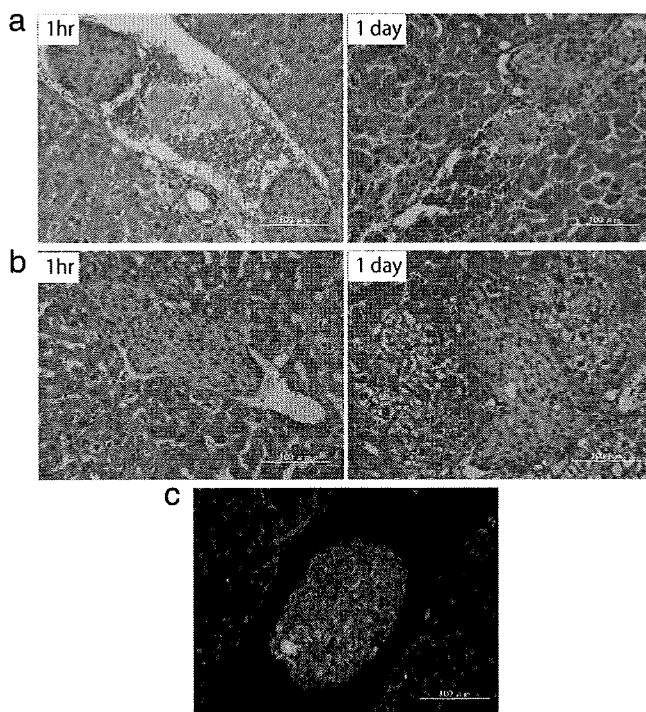


FIGURE 4. Histochemical analysis of (a) control islets and (b) poly(ethylene glycol) islets transplanted into the liver of diabetic mice through the portal vein by hematoxylin-eosin staining, and (c) poly(ethylene glycol) islets by insulin staining. These grafts were retrieved at 1 hr and 1 day postintraportal 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

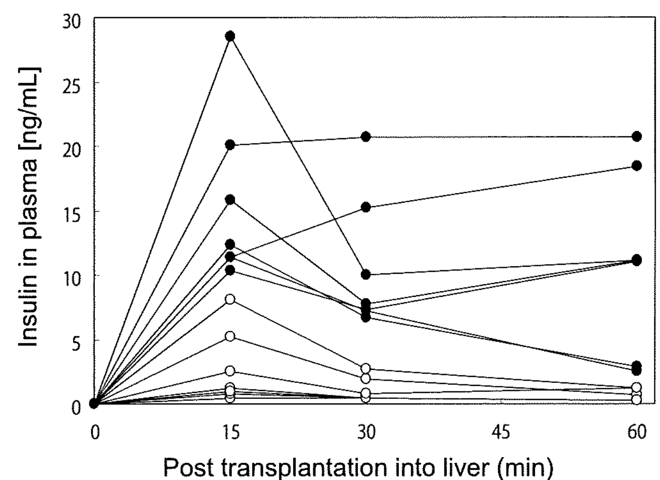


FIGURE 5. Changes in the blood insulin levels of diabetic mice after intraportal transplantation of control islets (*closed circles*, n=7) or PEG islets (*open circles*, n=7). The blood insulin levels were determined by ELISA.

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

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.

REFERENCES

1. Shapiro AM, Lakey JR, Ryan EA, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* 2000; 343: 230.
2. Ryan EA, Lakey JR, Rajotte RV, et al. Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol. *Diabetes* 2001; 50: 710.
3. Moberg L, Johansson H, Lukinius A, et al. Production of tissue factor by pancreatic islet cells as a trigger of detrimental thrombotic reactions in clinical islet transplantation. *Lancet* 2002; 360: 2039.
4. Johansson H, Lukinius A, Moberg L, et al. Tissue factor produced by the endocrine cells of the islets of Langerhans is associated with a negative outcome of clinical islet transplantation. *Diabetes* 2005; 54: 1755.
5. van der Windt DJ, Bottino R, Casu A, et al. Rapid loss of intraportally transplanted islets: An overview of pathophysiology and preventive strategies. *Xenotransplantation* 2007; 14: 288.
6. Ozmen L, Ekdahl KN, Elgue G, et al. Inhibition of thrombin abrogates the instant blood-mediated inflammatory reaction triggered by isolated human islets: Possible application of the thrombin inhibitor melagatran in clinical islet transplantation. *Diabetes* 2002; 51: 1779.
7. Johansson H, Goto M, Dufrane D, et al. Low molecular weight dextran sulfate: A strong candidate drug to block IBMIR in clinical islet transplantation. *Am J Transplant* 2006; 6: 305.

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

8. Contreras JL, Xie D, Mays J, et al. A novel approach to xenotransplantation combining surface engineering and genetic modification of isolated adult porcine islets. *Surgery* 2004; 136: 537.
9. Cabric S, Sanchez J, Lundgren T, et al. Islet surface heparinization prevents the instant blood-mediated inflammatory reaction in islet transplantation. *Diabetes* 2007; 56: 2008.
10. Teramura Y, Iwata H. Islets surface modification prevents blood-mediated inflammatory responses. *Bioconjug Chem* 2008; 19: 1389.
11. Totani T, Teramura Y, Iwata H. Immobilization of urokinase to islet surface by amphiphilic poly (vinyl alcohol) carrying alkyl side chains. *Biomaterials* 2008; 29: 2878.
12. Miura S, Teramura Y, Iwata H. Encapsulation of islets with ultra-thin polyion complex membrane through poly(ethylene glycol)-phospholipids anchored to cell membrane. *Biomaterials* 2006; 27: 5828.
13. Teramura Y, Kaneda Y, Iwata H. Islet-encapsulation in ultra-thin layer-by-layer membranes of poly(vinyl alcohol) anchored to poly-(ethylene glycol)-lipids in the cell membrane. *Biomaterials* 2007; 28: 4818.
14. Teramura Y, Kaneda Y, Totani T, et al. Behavior of synthetic polymers immobilized on cell membrane. *Biomaterials* 2008; 29: 1345.
15. Iwata H, Takagi T, Shimizu H, et al. Agarose for bioartificial pancreas. *J Biomed Mater Res* 1992; 26: 967.
16. Lee DY, Park SJ, Nam JH, et al. A new strategy toward improving immunoprotection in cell therapy for diabetes mellitus: Long-functioning PEGylated islets in vivo. *Tissue Eng* 2006; 12: 615.
17. Yun Lee D, Hee Nam J, Byun Y. Functional and histological evaluation of transplanted pancreatic islets immunoprotected by PEGylation and cyclosporine for 1 year. *Biomaterials* 2007; 28: 1957.
18. Goto M, Johansson H, Maeda A, et al. Low molecular weight dextran sulfate prevents the instant blood-mediated inflammatory reaction induced by adult porcine islets. *Transplantation* 2004; 77: 741.
19. Zalipsky S. Functionalized poly(ethylene glycol) for preparation of biologically relevant conjugates. *Bioconjug Chem* 1995; 6: 150.
20. Klibanov AL, Maruyama K, Torchilin VP, et al. Amphipatic poly(ethylene glycol)s effectively prolong the circulation time of liposome. *FEBS Lett* 1990; 268: 235.
21. Bradley AJ, Devine DV, Ansell SM, et al. Inhibition of liposome-induced complement activation by incorporated poly(ethylene glycol)-lipids. *Arch Biochem Biophys* 1998; 357: 185.
22. Chiu GN, Bally MB, Mayer LD. Effects of phosphatidylserine on membrane incorporation and surface protection properties of exchangeable poly(ethylene glycol)-conjugated lipids. *Biochim Biophys Acta* 2002; 18: 37.
23. Arima Y, Toda M, Iwata H. Complement activation on surfaces modified with ethylene glycol units. *Biomaterials* 2008; 29: 551.

e-TOCs and e-Alerts

Receive the latest developments in transplantation as soon as they're available.

Request the delivery of *Transplantation's* e-Alerts directly to your email address. This is a fast, easy, and free service to all subscribers. You will receive:

- Notice of all new issues of *Transplantation*, including the posting of new issues at the *Transplantation* website
- Complete Table of Contents for all new issues

Visit www.transplantjournal.com and click on e-Alerts.

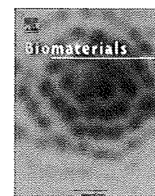
Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

Biomaterials 31 (2010) 2229–2235

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials



Control of cell attachment through polyDNA hybridization

Yuji Teramura [a], Hao Chen [b], Takuo Kawamoto [a], Hiroo Iwata [b,*]

[a] *Radioisotope Research Center, Kyoto University, Yoshida-Konoe-Cho, Sakyo-ku, Kyoto, 606-8501, Japan*
[b] *Department of Reparative Materials, Institute for Frontier Medical Sciences, Kyoto University, 53 Kawara-Cho, Shogoin, Sakyo-ku, Kyoto, 606-8507, Japan*

ARTICLE INFO

Article history:
Received 10 November 2009
Accepted 26 November 2009
Available online 11 December 2009

Keywords:
Cell–cell attachment
Poly(ethylene glycol)-lipid (PEG-lipid)
DNA hybridization
Surface modification

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.

© 2009 Elsevier Ltd. All rights reserved.

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*-

* Corresponding author. Tel./fax: +81 75 751 4119.
E-mail address: iwata@frontier.kyoto-u.ac.jp (H. Iwata).

0142-9612/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2009.11.098