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Regenerative Therapy 8 (2018) 65–72

Contents lists available at ScienceDirect

Regenerative Therapy

journal homepage: http://www.elsevier.com/locate/reth

Original Article

The liver surface as a favorable site for islet cell sheet transplantation in type 1 diabetes model mice

Izumi Fujita [a, b], Rie Utoh [a, c], Masakazu Yamamoto [b], Teruo Okano [a], Masayuki Yamato [a, *]

[a] Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan
[b] Department of Surgery, Institute of Gastroenterology, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan
[c] Department of Applied Chemistry and Biotechnology, Graduate School of Engineering, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan

ARTICLE INFO

Article history:
Received 25 January 2018
Received in revised form
19 March 2018
Accepted 12 April 2018

Keywords:
Cell sheet
Islet cells
Type 1 diabetes mellitus
Transplantation site
Cellular therapy

ABSTRACT

Introduction: Islet transplantation is one of the most promising therapeutic approaches for patients with severe type 1 diabetes mellitus (T1DM). Transplantation of engineered islet cell sheets holds great potential for treating T1DM as it enables the creation of stable neo-islet tissues. However, a large mass of islet cell sheets is required for the subcutaneous transplantation to reverse hyperglycemia in diabetic mice. Here, we investigated whether the liver surface could serve as an alternative site for islet cell sheet transplantation.

Methods: Dispersed rat islet cells (0.8×10^6 cells) were cultured on laminin-332-coated thermoresponsive culture dishes. After 2 days of cultivation, we harvested the islet cell sheets by lowering the culture temperature using a support membrane with a gelatin gel. We transplanted two recovered islet cell sheets into the subcutaneous space or onto the liver surface of severe combined immunodeficiency (SCID) mice with streptozocin-induced diabetes.

Results: In the liver surface group, the non-fasting blood glucose level decreased rapidly within several days after transplantation. In marked contrast, the hyperglycemia state was maintained in the subcutaneous space transplantation group. The levels of rat C-peptide and insulin in the liver surface group were significantly higher than those in the subcutaneous space group. An immunohistological analysis confirmed that most of the islet cells engrafted on the liver surface were insulin-positive. The CD31-positive endothelial cells formed vascular networks within the neo-islets and in the surrounding tissues. In contrast, viable islet cells were not found in the subcutaneous space group.

Conclusions: Compared with the subcutaneous space, a relatively small mass of islet cell sheets was enough to achieve normoglycemia in diabetic mice when the liver surface was selected as the transplantation site. Our results demonstrate that the optimization of the transplantation site for islet cell sheets leads to significant improvements in the therapeutic efficiency for T1DM.

1. Introduction

Type 1 diabetes mellitus (T1DM) is an autoimmune disease with progressive destruction of insulin-producing pancreatic β-cells. Individuals with T1DM need lifelong administrations of exogenous insulin through multiple daily subcutaneous injections. In some individuals with severe T1DM, it is very difficult to achieve better glycemic control by insulin administrations, and such individuals thus experience repeated hypoglycemic episodes and occasionally develop fatal hypoglycemia. Islet transplantation has high potential for the treatment of patients with severe T1DM as an alternative therapy to insulin replacement or whole pancreas transplantation.

Abbreviations: T1DM, type 1 diabetes mellitus; SCID mouse, severe combined immunodeficient mouse; IBMIR, instant blood-mediated inflammatory reaction; STZ, streptozocin; TRD, temperature-responsive culture dish; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; IPGTT, intraperitoneal glucose tolerance test; MSC, mesenchymal stem cell.

* Corresponding author. Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan. Fax: +81 3 3359 6046.
E-mail address: yamato.masayuki@twmu.ac.jp (M. Yamato).

Peer review under responsibility of the Japanese Society for Regenerative Medicine.

https://doi.org/10.1016/j.reth.2018.04.002

In 2000, Shapiro and colleagues succeeded in achieving insulin independence in all seven patients with T1DM using a glucocorticoid-free immunosuppressive regimen after the infusion of islets into the portal vein of the patients [1]. This approach, the so-called Edmonton protocol, attracted worldwide attention as a therapeutic breakthrough in islet transplantation. Since then, much progress in the islet manufacturing processes and immunosuppression regimens has been made, resulting in significant improvements in the rates of 5-year insulin independence from only approx. 10% [2] to >50% [3].

In clinical studies, the intrahepatic transplantation of islets through the portal vein is the most prevalent method, with minimal invasiveness. Physiologically, endogenous insulin secreted from the pancreas flows primarily into the liver through the portal vein and is consumed there, and then acts on muscle and adipose tissues. The non-physiological insulin distribution by subcutaneous injections therefore causes inadequate glycemic control in T1DM patients [4]. In contrast, for insulin secreted from the transplanted intrahepatic islets, it is possible to reproduce the physiological insulin action [5,6].

The liver as a transplantation site enables an efficient supply of oxygen and nutrition from the hepatic sinusoids to the transplanted islets. However, there is a critical weakness in the intrahepatic islet transplantation; in a study of this technique, approx. 60% of the transplanted islets were lost in the very early stage of the post-transplantation period [7]. Instant blood-mediated inflammatory reaction (IBMIR) is a nonspecific immune reaction triggered by the activation of coagulation factors and the complement system through a direct exposure of the graft cells to blood [8,9], which causes the loss of the transplanted islets. Islets isolated from multiple donors were therefore required to achieve normoglycemia in the majority of clinical islet-transplantation trials [3], and this has hampered the standardization and wide adaptation of the current islet transplantation therapies for T1DM.

Our research group has developed cell sheet technology for regenerative therapy and tissue engineering. We prepared contiguous monolayer cell sheets using temperature-responsive cell culture dishes in a noninvasive manner [10], which could be directly transplanted to host tissues (e.g., liver, heart, subcutaneous space, cornea, esophagus, and periodontal ligaments) without necessitating adhesive agents [11,12]. In fact, cell sheet technology has been applied in several clinical trials including those for regeneration therapies of myocardial tissue, cornea, esophageal mucosa, lung, and periodontal ligament [13–18].

We reported a new proof-of-concept therapeutic approach to create functional neo-islet tissues in the subcutaneous space of diabetic mice by transplanting islet cell sheets [19,20]. This approach has several advantages for islet transplantation in terms of the potentially lesser IBMIR, high accessibility to the grafts, and less-invasive transplantation procedure. However, there is a critical problem regarding islet cell sheet transplantation in the subcutaneous space; a large mass of islet cells (total 1.1×10^7 cells per mouse) was required to achieve euglycemia in streptozocin (STZ)-induced diabetic model mice. The inadequate vascularization and the poor supply of blood flow in the subcutaneous space [21] may not be suitable for islet cell sheet transplantation, because the oxygen supply limitation causes a restriction of islet cell survival and function [22–24]. The nonphysiological action of insulin secreted from the subcutaneous space was also considered to be problematic.

In the present study, we investigated whether there is an alternative, more favorable place for islet cell sheet transplantation. We selected the liver surface as a new transplantation site, where the transplanted cell sheets could be indirectly but effectively exposed to the blood supply. We performed the transplantation of islet cell sheets onto the liver surface of diabetic mice and compared the transplantation efficiency and therapeutic values between the liver surface group and subcutaneous space group.

2. Materials and methods

2.1. Animals

All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of Tokyo Women's Medical University. Male Lewis rats, 8–12 weeks of age (LEW/CrlCrlj, Charles River, Yokohama, Japan) were used as donors for islet cell sheet transplantation. Male severe combined immunodeficient (SCID) mice, 7–10 weeks of age (C.B-17/lcr-scid/scidJcl, CLEA Japan, Tokyo) were used as recipients. Diabetes was induced in the SCID mice by a single intraperitoneal injection of streptozocin (220 mg/kg body weight). Non-fasting blood glucose levels were measured in blood samples collected from the tail vein of each mouse with the use of a handheld blood glucose meter (Glutest Neo Super; Sanwa Chemistry Laboratory, Nagoya, Japan). SCID mice with hyperglycemia greater than a non-fasting blood glucose level of 350 mg/dL were used for the transplantation of islet cell sheets. All animals were kept under a controlled environment (22–24 °C, 55 ± 10% humidity, and a 12-h light/dark cycle).

2.2. Islet isolation and preparation of single islet cells

We isolated pancreas islets from Lewis rats by collagenase digestion as described [20,24] with slight modifications. Each pancreas tissue was swollen by an injection of 2 mg/mL collagenase type V (Sigma–Aldrich, St. Louis, MO) in Hanks' balanced salt solution (Sigma–Aldrich) through the common bile duct. The harvested pancreas was further digested at 37 °C for 12 min. Subsequently, islets were purified by discontinuous gradient centrifugation using 1.119, 1.100, and 1.077 g/mL Histopaque (Sigma–Aldrich) and then cultured overnight in the cell culture medium, RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS; Japan Bio Serum, Hiroshima, Japan), 5.5 mmol/L glucose (Thermo Fisher Scientific), 10 mmol/L HEPES (Thermo Fisher Scientific), 1 mmol/L sodium pyruvate (Thermo Fisher Scientific), 0.1 mmol/L nonessential amino acids (Thermo Fisher Scientific), 2 mmol/L GlutaMAX-I (Thermo Fisher Scientific), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Sigma–Aldrich) at 37 °C in 5% CO_2. Islets were treated with 0.125% trypsin–EDTA solution (Thermo Fisher Scientific) to obtain single cell suspensions. The viability of dispersed islet cells was assessed by a trypan blue dye exclusion test. The dispersed islet cells with a viability exceeding 90% were used for preparing islet cell sheets.

2.3. Fabrication of islet cell sheets

Single islet cells were cultured on temperature-responsive culture dishes (TRDs) to obtain monolayer cell sheets as described [19,20]. The 24-well cell culture plates were covalently grafted with a triple amount of the temperature-responsive polymer [poly(*N*-isopropylacrylamide)] compared to that of commercially available dishes (UpCell; CellSeed, Tokyo) and were subsequently coated with human laminin-332 (0.2 μg/cm^2; ReproCELL, Yokohama, Japan) at 37 °C for 2 h to promote the attachment of islet cells [25]. Subsequently, a 10 mm × 10 mm cloning cylinder (Merck Millipore, Billerica, MA) was placed in each well. The islet cells were seeded at 0.8×10^6 cells, which correspond to the number of cells composing ~850 IEQ (islet equivalents) [26], per well into the cloning cylinders placed on the laminin-332 coated TRDs. Islet cells were cultured to confluence for 2 days in the cell culture medium.

2.4. Recovery of islet cell sheets by the in situ gelation method

To transfer islet cell sheets onto the liver surface and the subcutaneous space in mice, we recovered islet cell sheets from TRDs using an in situ gelation method as described [27] with slight modifications. Gelatin powder (RM-100; Jellice, Miyagi, Japan) was dissolved in Hanks' balanced salt solution (Sigma–Aldrich) at a concentration of 16% (w/v) at 37 °C for 60 min. The gelatin solution was kept at 37 °C in a water bath until its use in experiments. The cloning cylinder and the culture medium were removed from TRDs at 2 days after the inoculation of the islet cells. The gelatin solution was poured onto each culture dish. Thereafter, a supporting membrane (Cell Shifter; CellSeed) was placed onto the gelatin solution, and the culture plate was incubated at 20 °C for 60 min to promote the gelation of the gelatin solution. The membrane with the gelatin gel attached to an islet cell sheet was slowly removed from TRDs for transplantation (Fig. 1).

2.5. Transplantation of islet cell sheets

Under anesthesia using isoflurane, two sheets of the islet cells (total 1.6×10^6 cells) were transplanted onto the liver surface (n = 7) or into the subcutaneous space (n = 7) of STZ-induced diabetic SCID mice. In the liver surface transplantation, we gently scratched a 10-mm^2 area on the surface of the left lateral lobe of the recipient liver with a cotton swab to remove the liver capsule before transplanting the islet cell sheets. After hemostasis was achieved by applying pressure with cotton swabs, an islet cell sheet with the support membrane and the gelatin gel was transplanted onto the scratched liver surface. Subsequently, the support membrane was removed slowly from the gelatin gel. The gelatin gel was liquefied by the body temperature of mouse shortly after transplantation. After the gelatin gel melted, these steps were repeated once for the subsequent transplantation of another islet sheet. Lastly, two islet cell sheets on the left lateral lobe of the liver were covered by the median lobe of the liver. It is notable that the experimental results for the cell sheet transplantation onto the scratched liver surface were more reproducible compared with those onto the intact liver surface.

In the subcutaneous space transplantation, an L-shaped incision was made on the left side of the abdomen of the recipient mouse, and a subcutaneous space was created by exfoliating the layers of skin. An islet cell sheet with the support membrane and the gelatin gel was transplanted on the superficial fascial layer in the subcutaneous space. The support membrane was then removed from the gelatin gel. Another islet cell sheet was transplanted on the top of the transplanted islet cell sheet in the same way (Fig. 1).

2.6. Measurement of serum insulin and C-peptide

Mouse blood samples were obtained at days 0, 14, and 28. The levels of serum insulin, rat-specific C-peptide, and mouse-specific C-peptide were measured using the Rat Insulin ELISA kit

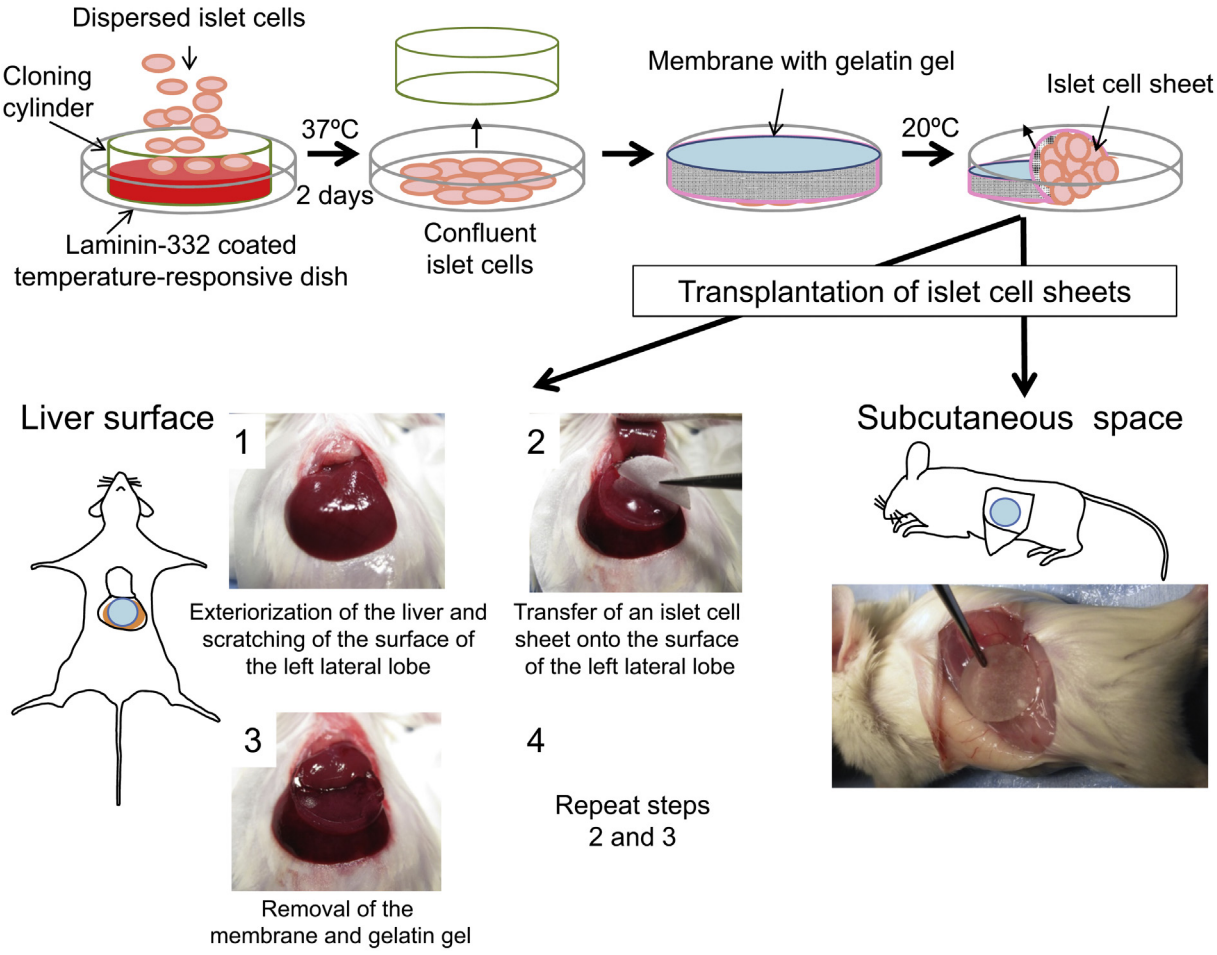

Fig. 1. Schematic of the recovery and transplantation of rat islet cell sheets from a temperature-responsive dish (TRD) using a gelatin/membrane. Dispersed rat islet cells were seeded into a cloning cylinder placed on a laminin-332 coated TRD and cultured for 2 days at 37 °C. The islet cell sheets were recovered from TRDs using gelatin/membranes and transplanted onto the liver surface or the subcutaneous spaces of STZ-induced diabetic SCID mice.

(Mercodia, Uppsala, Sweden), the Rat C-peptide ELISA kit (Mercodi), and the Mouse C-peptide ELISA kit (Mercodi) according to the manufacturer's instruction, respectively. The Rat Insulin Elisa kit shows cross-reactivity with mouse insulin.

2.7. Intraperitoneal glucose tolerance tests

To evaluate the functionality of the transplanted islet cell sheets, we conducted the intraperitoneal glucose tolerance test on day 28. After 6 h of fasting, mice were inoculated with a glucose solution (0.22 mg/g body weight) intraperitoneally. Blood samples were collected at 0, 15, 30, 60, 90 and 120 min after the glucose administration.

2.8. Histochemical analysis

Tissue samples from dissected mice at day 28 were fixed with 4% paraformaldehyde solution (Wako, Osaka, Japan) overnight and embedded in paraffin, and we used a microtome to prepare 5-μm-thick sections for hematoxylin–eosin (H&E) staining and an immunohistochemical analysis. For the immunohistochemical analysis, the sections were incubated with 10% goat serum for 30 min and then incubated with guinea pig polyclonal anti-insulin antibody, mouse monoclonal anti-glucagon antibody, rabbit polyclonal anti-CD31 antibody, or rabbit monoclonal anti-Ki67 antibody (all from Abcam, Cambridge, MA) overnight. The bound antibodies were detected by Alexa Fluor 488-conjugated anti-guinea pig IgG, Alexa Fluor 594-conjugated anti-mouse IgG, or Alexa Fluor 594-conjugated anti-rabbit IgG (Thermo Fisher Scientific) as secondary antibodies. The stained sections were mounted using ProLong gold antifade reagent with DAPI (Thermo Fisher Scientific). The Ki67-labeling index of the β cells in the neo-islet tissues was determined by calculating the ratio of Ki67-positive nuclei to insulin^{+}-β cells in randomly selected fields of liver sections from 5 individual mice ($n = 5$).

2.9. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method

Five-μm paraffin sections were double-stained by the TUNEL method for apoptotic nuclei and by immunohistochemistry of insulin for β cells. Apoptotic nuclei were detected using ApopTag Red In Situ Apoptosis Detection Kit (Merck Millipore) according to the manufacturer's instructions. Subsequently, immunohistochemistry for insulin was performed using guinea pig polyclonal anti-insulin antibody (Abcam).

2.10. Statistical analysis

All data are presented as the mean ± standard error of the mean (SEM). Statistical analyses were performed using IBM SPSS Statistics 18 software (IBM Japan, Tokyo). For comparisons between two groups, Student's *t*-test or the Mann–Whitney *U*-test was used. The statistical significance of differences among three or more groups was determined by an analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) or Games Howell post-hoc test. The statistical significance of differences is indicated by asterisks ($^{*}p < 0.05$).

3. Results

3.1. Transplantation efficacy of islet cell sheets onto the liver surface

We harvested the rat islet cell sheets from the TRDs by using the support membrane with the gelatin gel (Fig. 1). For the comparison of the transplantation efficiencies of the islet cell sheets for the liver surface and the subcutaneous space groups, the same amount of islet cells (two cell sheets with a total of 1.6×10^6 cells) were transplanted. Cell sheets were transplanted onto the surface of the capsule-removed liver surface or into the subcutaneous space of diabetic SCID mice. The level of nonfasting blood glucose in the liver surface group ($n = 7$) achieved normoglycemia within a few days after transplantation, and it was maintained at least for 28 days (Fig. 2A). In marked contrast, the state of hyperglycemia was maintained in the subcutaneous space group. The blood glucose level remained at >300 mg/dL in the subcutaneous group ($n = 7$), although this value was slightly and temporary decreased at 2–7 days after transplantation in comparison with that in the non-transplanted control group.

The body weight of the mice gradually increased in the liver surface group, indicating that good glycemic control was achieved (Fig. 2B). By contrast, restoration of the body weight was prevented in both the nontransplanted group and the subcutaneous space group.

3.2. Insulin and c-peptide secretory capabilities of transplanted islet cell sheets

The serum insulin concentration was extremely low (approx. 0.3 μg/L) in the diabetic SCID mice before the islet cell sheets were transplanted (Fig. 3A). In the liver surface group, the serum insulin level was significantly increased to a level similar to that of the normal SCID mice (1.19 ± 0.11 μg/L) after transplantation. The serum insulin concentrations at days 14 and 28 were 1.37 ± 0.43 μg/L and 1.57 ± 0.23 μg/L, respectively. The subcutaneous space group did not show a change in the serum insulin concentration compared to the pre-transplant level. The insulin levels remained consistently low for at least 28 days after transplantation.

Likewise, the levels of rat C-peptide in the subcutaneous space group at days 14 and 28 were significantly lower than those of the liver surface group, although a slight increase of the rat c-peptide level was observed (especially at day 14). These results clearly suggested that the transplantation of islet cell sheets onto the liver surface is more effective than that in the subcutaneous space, in terms of insulin secretion and the subsequent achievement of normoglycemia.

3.3. Functional assessment of transplanted islet cell sheets

To assess the glucose response ability of the transplanted islet cell sheets in the diabetic mice, we performed an intraperitoneal glucose tolerance test (IPGTT) at day 28 (Fig. 4). In the subcutaneous space group, the blood glucose level consistently remained high (>400 mg/dL) after glucose administration, as was the case for the nontransplanted diabetic mice. In the liver surface group, the blood glucose level reached the maximum (285.2 ± 38.2 mg/dL) at 30 min after glucose administration and then gradually decreased to the normal levels within approx. 120 min. The glucose response curve showed a pattern similar to that of the normal SCID mice, indicating that the transplanted islet cell sheets on the liver surface were functional and capable of secreting an amount of insulin that was sufficient to achieve normoglycemia in response to glucose administration.

3.4. Histological assessment of the transplanted islet cell sheets in the liver

We performed a histological assessment on day 28 after transplantation to investigate the graft survival and revascularization in the neo-islets created on the liver surface (Fig. 5),

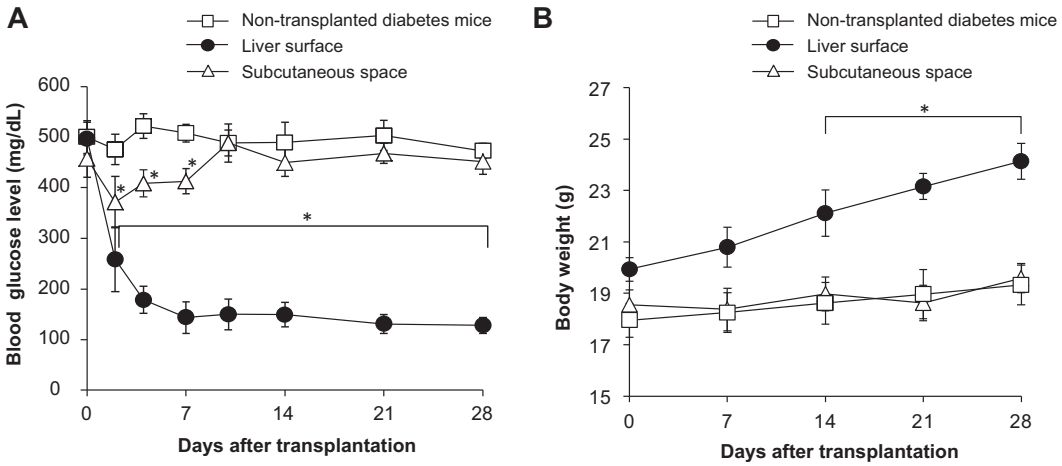

Fig. 2. Measurement of the blood glucose level and body weight of diabetic mice after islet cell sheet transplantation. (A, B) Fasting blood glucose levels (A) and body weights (B) in diabetic SCID mice. Two islet cell sheets (total 1.6×10^6 cells) were transplanted onto the surface of the liver (*circles*: n = 7) or into the subcutaneous space (*triangles*: n = 7) of STZ-induced diabetic SCID mice. The blood glucose level and body weight in the diabetic SCID mice without the transplantation of islet cell sheets was also monitored as a control (*squares*: n = 7). Data are mean ± SEM. *p < 0.05 vs. non-transplanted control.

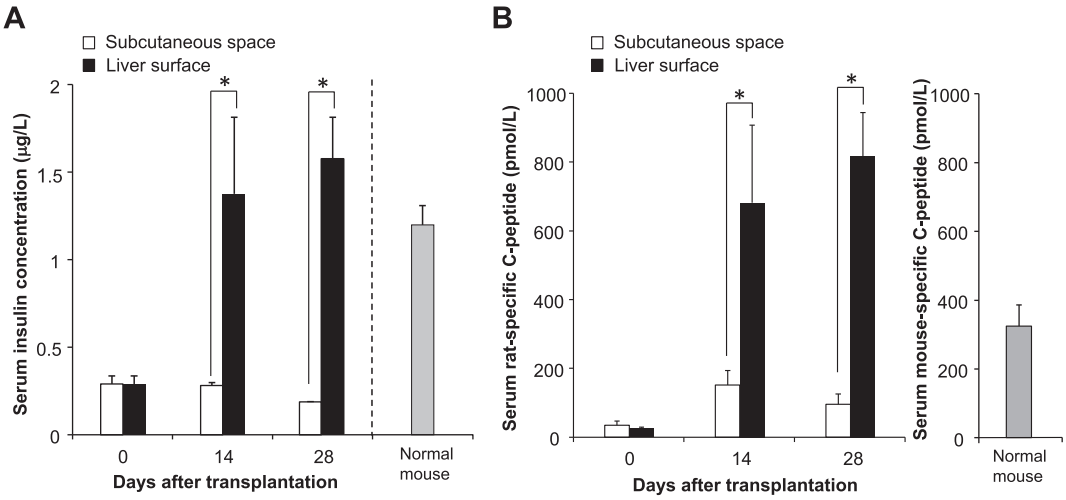

Fig. 3. The serum concentrations of insulin and rat-specific C-peptide in diabetic mice after islet cell sheet transplantation. Islet cell sheets were transplanted onto the surface of the liver or into the subcutaneous space of STZ-induced diabetic SCID mice. (A, B) The serum concentration of insulin (A) and rat-specific C-peptide (B) of diabetic SCID mice in the liver surface (*black bar*: n = 7) and subcutaneous (*white bar*: n = 7) transplantation groups was measured by ELISA at 2 days prior to and 14 and 28 days after transplantation. The concentrations of insulin and mouse-specific C-peptide in normal SCID mice (*gray bar*: n = 7) were determined as a control. Data are mean ± SEM. *p < 0.05 vs. the subcutaneous space group.

because revascularization is a significantly important factor for improving graft survival and islet cell functions [28–30]. The histological examination confirmed that the transplanted islet cell sheets were effectively engrafted between two liver lobes (Fig. 5A, B), forming a neo-islet tissue with a thickness of 50–70 μm. In the experimental procedure, the islet cell sheets transplanted on the decapsulated surface of the left lateral lobe of the liver were subsequently covered by the median lobe of liver at the time of transplantation (Fig. 1), which prevented the adhesion of the islet cell sheets to the parietal peritoneum. The decapsulation of the liver promoted the engraftment of islet cell sheets to the liver surface (data not shown).

In contrast to the native islet morphology, the engineered neo-islet tissue between the liver lobes was horizontally long (Fig. 5A, B) and consisted predominantly of insulin-positive β-cells (Fig. 5C). Glucagon-positive α-cells were also present, but their number was small and they were sparsely distributed in the neo-islet tissue. CD31-positive vascular networks containing blood cells were formed in the neo-islet tissues at day 28 (Fig. 5B, D). β cell proliferation and apoptosis were assessed by the double staining for insulin/Ki67 and TUNEL staining, respectively. As a result, 0.95 ± 0.41% (n = 5) and 0.06 ± 0.06% (n = 5) of the insulin$^+$ cells were Ki-67- (Fig. 5E, F) and TUNEL-positive on day 28, respectively. The ratios of Ki-67$^+$ and TUNEL$^+$ cells were almost equal to those of the β cells in normal pancreases [7,31]. These results, together with the results on the levels of rat-specific C-peptide virtually unchanged from day 14–28 (Fig. 3B), suggest the stable engraftment of the transplanted islet cell sheets. Conversely, it was difficult to find transplanted islet cells in the subcutaneous space group at day 28 (data not shown), suggesting that the grafts did not survive well in the subcutaneous space. These results revealed that compared to the subcutaneous space, a small mass of islet cells (total 1.6×10^6 cells) could be engrafted and survived for a long period on the liver surface.

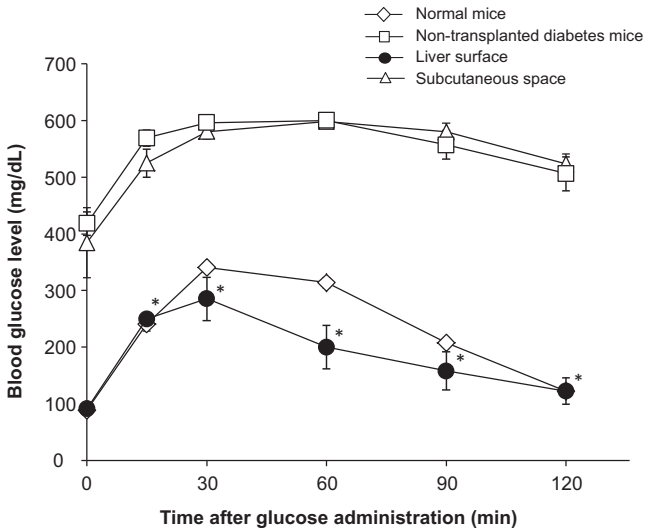

Fig. 4. IGTT results after islet cell sheet transplantation. Islet cell sheets were transplanted onto the surface of the liver or into the subcutaneous space of diabetic SCID mice. Glucose was injected intraperitoneally into nontransplanted normal (*squares*: n = 7) and diabetic SCID mice (*diamonds*: n = 7), transplanted diabetic SCID mice (subcutaneous, *triangles*: n = 7; liver surface, *circles*: n = 7) on day 28. The blood glucose level was monitored up to 120 min after glucose administration. Data are mean ± SEM. $^{*}p < 0.05$ vs. non-transplanted diabetic SCID mice.

4. Discussion

We reported an approach for creating neo-islet tissues in the subcutaneous space of diabetic mice by transplanting islet cell sheets prepared from dispersed islet cells [20]. However, a large mass of islet cells (total 1.1×10^7 cells per mouse) was required to achieve euglycemia in mice with STZ-induced diabetes. The most likely reason for this insufficiency was that the transplanted cells were neither viable nor functional because of the limited supply of oxygen and nutrition caused by the lack of early neovascularization [32]. In the present study, we selected the liver surface as a new site for transplanting islet cell sheets for treating diabetic mice. Euglycemia was successfully achieved with a lesser amount of islet cells (total 1.6×10^6 cells per mouse) when the liver surface was used compared to the transplantation into the subcutaneous space.

The immunohistochemical analysis showed that the vascularized neo-islet tissues were formed on the liver surface, which may have primarily contributed to the preservation of islet cell function and viability. In marked contrast, viable islet cells could not be found in the subcutaneous space group. In the subcutaneous space transplantation, the inherent limitations including the lack of early vascularization, induction of local inflammation, and mechanical stress on the graft [33] potentially hampered the survival and engraftment of the transplanted islet cell sheets prepared from 1.6×10^6 islet cells.

The intraportal transplantation of islets to the liver has been the most prevalent, safe, and less-invasive procedure. Optimal sites for islet transplantation have been explored to maximize the function and survival of islet cells. In addition to the liver, researchers have attempted to transplant islets to various sites including the pancreas, kidney subcapsular space, subcutaneous space, spleen, omental pouch, gastric submucosa, and peritoneal cavity [5,6,32]. A reversal of hyperglycemia was achieved by using a smaller number of islets when the liver, spleen, and kidney subcapsular space were used, compared with other sites.

However, kidney and spleen as the transplantation sites do not have substantial therapeutic advantages over the liver. First, the kidney may not be metabolically suitable, because insulin secreted from the kidney does not reproduce its physiological behavior. Moreover, the transplantation of islets under the kidney capsule is a significantly invasive operation. Splenic site remains at risk of hemorrhage at the time of islet transplantation as well as the high accessibility of lymphocytes to the graft. For these reasons, an intrahepatic site has been considered to be particularly suitable for islets transplantation. However, as mentioned above, multiple donors are usually required to achieve insulin independence by the intraportal transplantation procedure. Not only IBMIR, but also graft hypoxia and the liver immune system are critical problems [34]. In addition, the transplantable cell volume is limited in order to avoid the acute portal thrombosis in the intraportal transplantation protocol [35].

The liver surface is an attractive alternative site for islet transplantation as it allows for the prevention of IBMIR by avoiding the direct infusion of the blood flow to the transplanted islets or islet cells. The transplantation of islets or islet cells onto the liver surface can potentially reduce the required mass of islet cells to reverse hyperglycemia, but it has not been possible to stably immobilize dispersed cells or cell aggregates onto the surface. Cell sheet technology enables us to directly and stably transplant a cellular construct to the surface of host tissues, with the help of the extracellular matrix layer accumulated on the surface of the cell sheets [11], which works as a natural glue.

Cell sheets composed of fibroblasts, mesenchymal stem cells (MSCs), and iPSC-derived hepatocytes have been transplanted to the liver surface of mice [27,36,37]. The liver surface is advantageous in terms of the high accessibility and the lesser invasiveness with the use of a laparoscope. In addition, it would be possible to remove the transplanted tissues on the liver surface by employing radio-frequency, microwave, or laser ablation with a minimally invasive procedure [38] when problems arise in the transplanted cells. These are the primary reasons why we tested the liver surface for islet cell sheet transplantation.

Islets are highly vascularized tissues in vivo, and the inner vasculature is essential for effectively secreting insulin in response to the blood glucose level [39]. Vascular connections between the interior structure of islets and the surrounding tissues are severed at the time of islet isolation by collagenase treatment. Usually, revascularization for transplanted islets begins several days after transplantation and is established within 10–14 days [40]. Nutrients and oxygen are supplied solely by diffusion from the surrounding tissue to the transplanted islets until the restoration of vascularization is completed. Limitations of oxygen and nutrition supply cause the degradation of cell functions and the poor survival of the transplanted islets. Small islets, typically with sizes less than 100 μm, are therefore superior to larger islets, in both in vivo transplantation and in vitro cultivation [24,41–43].

In our experiments, on the other hand, revascularization to the transplanted islet cell sheets was significantly promoted in the liver surface group, forming densely vascularized neo-islet tissues. This revascularization would have been accelerated by the geometric characteristics of the thin cell sheets prepared using TRDs, with the help of the effective interaction with the host liver surface. Capillary networks formed in the islet cell sheets may also have contributed to the enhancement of cell survival, function, and engraftment.

We successfully transplanted islet cell sheets onto the liver surface of diabetic mice with the combination of an efficient recovery method by the in situ gelation of gelatin [27]. This in situ gelation method improved the recovery efficiency of the islet cells from TRDs compared to the conventional methods using the membrane only [19,20]. However, a portion of the adhered islet cells, at most ~20% of the cells, sometimes remained on and could

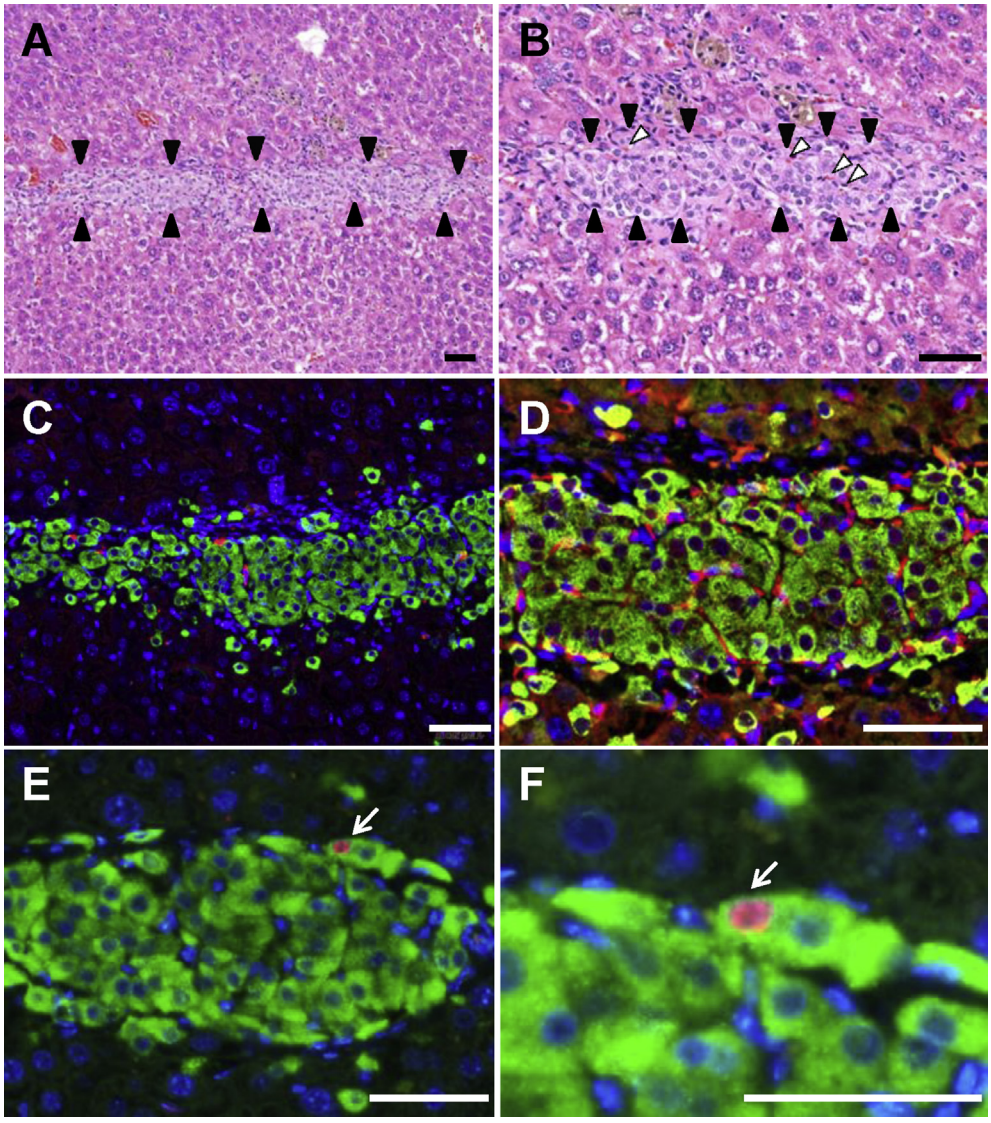

Fig. 5. Histological analysis of the islet cell sheets that were engrafted on the surface of the left lateral lobe of the liver and covered by the median lobe of the liver. (A, B) HE staining of neo-islet tissues between liver lobes on day 28. Neo-islet tissue (*black arrow heads*) and vascular networks containing blood cells (*white arrow heads*) are shown. (C) Double immunohistochemical staining for insulin (*green*) and glucagon (*red*) of neo-islet tissues in liver on day 28. (D) Double immunohistochemical staining for insulin (*green*) and CD31 (*red*) on day 28. (E, F) Double immunohistochemical staining for insulin (*green*) and Ki-67 (*red*) on day 28. Arrows denote Ki-67/insulin double positive β cells in the neo-islet tissue. Bar: 50 μm.

not be recovered from the TRDs (data not shown) because of the strong attachment of the islet cells to the TRDs, even though we used TRDs with a higher amount of grafted temperature-responsive polymer, poly(N-isopropylacrylamide). This suggested that an improvement in the recovery and transfer methods of islet cell sheets enables a further reduction of the required amount of islet cells to reverse hyperglycemia in the present liver surface transplantation procedure.

In a recent study, Hirabaru et al. created islet/MSC hybrid sheets by seeding whole islets onto a confluent layer of cells on MSCs [44]. Almost all of the cells were efficiently recovered from the TRDs, because the MSCs were completely detachable from the TRDs when the culture temperature was decreased to 20 °C. In addition, the MSC sheets promoted angiogenesis by secreting hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) at a subcutaneous site. The use of such hybrid cell sheets composed of dispersed islet cells and other types of cells may improve the recovery from TRDs and enhance the therapeutic efficacy of islet cell sheet transplantation.

5. Conclusions

Cell sheet technology enabled us to transplant islet cells to the liver surface, which was demonstrated herein to be an efficient and favorable site for islet cells. The islet cell sheets, prepared from a small number of dispersed islets (total 1.6×10^6 cells) and transplanted to the liver surface, normalized the blood glucose levels of diabetic mice, whereas this did not occur in the case of the subcutaneous space transplantation. Transplantation on the liver surface led to improved engraftment, revascularization, and a long-term survival of the islet cells, achieving euglycemia in STZ-induced diabetic mice, even though we used a relatively small mass of the islet cell sheets. The approach described herein may be useful as a new method for the islet cell-based treatment of T1DM.

Conflicts of interest

T. Okano, Ph.D is a founder and member of the board of CellSeed Inc. (Tokyo, Japan), which has licenses for certain cell sheet-related technologies and patents from Tokyo Women's Medical University. M. Yamato, Ph.D is a shareholder of CellSeed Inc. The other authors disclose no financial relationships relevant to this publication.

Acknowledgments

We thank Ms. Kazuko Kanegae for the technical assistance and Dr. Masumi Yamada for kindly editing the manuscript. This study was supported in part by the Creation of Innovation Centers for Advanced Interdisciplinary Research Areas Program, of the Project for Developing Innovation Systems “Cell Sheet Tissue Engineering Center (CSTEC)” (M.Y.) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, JSPS KAKENHI grant nos. 26350530 and 16J40041 (R.U.), and a research grant from Gastrointestinal Cancer Project funded by the Nakayama Cancer Research Institute (I.F.).

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