New Analytical Method for Pancreas and Liver Regeneration: Normalization of Streptozotocin-Induced Hyperglycemia by Retrograde Injection of Insulin Producing Cells

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Abstract. We established a new analytical system in which functioning cells were transplanted directly into the pancreas and liver. The retrograde transplantation of beta cell line, Min6 cells, into the streptozotocin-diabetic mice normalized plasma glucose and insulin levels. The injected cells were protected from pancreatic enzymes with enzyme inhibitor. Blood glucose decreased gradually over 10 days and the diabetic mice recovered weight at the same time. Intraperitoneal glucose tolerance test showed that the peak of plasma glucose of the transplanted mice was less than half that of the control. The insulin secretion of the transplanted mice was recovered and stimulated 4.6 times from the basal secretion. Histological analyses showed that the pancreas and liver were characterized by Min6 cell clusters dispersed throughout the organs. Min6 cells were detected near the pancreatic or bile ducts. It is suggested that the injected cells obstructed the peripheral ducts where they settled. The weight of pancreas and liver did not differ significantly in either Min6 transplanted or the control mice. The metabolic effects on the weights of these organs appeared the same in both groups. This is the first report that cells transplanted via ducts into the pancreas and liver performed their biological function. Our transplantation model makes possible the in vivo analysis of the regeneration machinery of the pancreas and liver.

Key words: TCPHI, Cholecystic duct, Pancreas, Liver


THERE is accumulating evidence that pancreatic or hepatic stem cells exist in the ducts where they function to repair damaged organs [1–6]. This supportive machinery for differentiation of the stem cells exists around the ducts, with differentiation being the result of chronological cell-cell interactions in these three-dimensional structures. As these complicated reactions cannot be reproduced completely in vitro, we established a new method to analyze in vivo cell interaction in which factor-secreting cells were transplanted around the ducts and analyzed for their functions. This incorporates the TCPHI (Trans-Cholecys-
tic Pancreateo-Hepato Injection) method which we previously established in a mouse model [7]. Although this retrograde approach is well known as endoscopic retrograde cholangiopancreatography [8], it is hardly ever used for cell transplantation. This is because the ducts are filled with digestive juices that destroy the injected cells, and subject them to a continuous flow that washes the cells away. Moreover, few experimental animals are suitable for cell transplantation into the pancreas. Experimental mice are also too small to perform transplantation, and there are few species of immunodeficient animals available for transplantation experiments.

In this paper, we devised a new method to transport biological materials into the pancreatic and hepatic ducts. The number of transplanted cells has to be sufficiently large to regulate the surrounding cells or body metabolism. In a previous study, we
only showed the qualitative analysis of retrograde transplantation [7]. We show here the quantitative results in which functional reconstruction of pancreas could be done by cell transplantation via the ducts. Insulin secreting Min6 cells [9, 10] were transplanted inside the pancreas and liver ducts of diabetic mice. This line grows slowly in vitro and secretes insulin depending on the concentration of blood sugar similar to that of normal beta cells. We analyzed whether Min6 cells could normalize the streptozotocin-induced hyperglycemia with the TCPHI method. This is the first report that the retrograde-transplanted cells around the ducts of the pancreas and liver were able to compensate for the pancreatic function.

Materials and Methods

Animals

The C57BL/6 Tg14 (act-EGFP) OsbY01 mice [11] were a gift from Dr. M. Okabe of Research Institute for Microbial Diseases, Osaka University and bred under specific pathogen-free conditions. Twelve week-old male mice weighing from 20 to 25 g were used in this study. To induce diabetes, normal recipient mice were treated with intraperitoneal injection of streptozotocin (STZ) at 200 mg/kg body weight (Sigma, St. Louis, MO, USA). STZ was dissolved with 50 mM citrate buffer pH 4.5 and injected every week until the mice had severe diabetes. Diabetes mellitus was confirmed by repetitive nonfasting blood glucose levels (more than 22 mM), polyuria and weight loss.

Cell

Min6 cell line is a gift from Dr. J. Miyazaki of Osaka University Medical School [9]. The cells were grown on collagen type I-coated dish (Iwaki, Chiba, Japan) in Dulbecco's modified Eagle's medium (DMEM, Iwaki, Chiba, Japan) supplemented with 15% heat inactivated fetal calf serum (FCS). The medium was changed every three to four days and the cells were regularly passed every two weeks. The cells were labeled with 1 µg/ml SP-Dil (Molecular Probes, Eugene, OR, USA) for a week. When the cells were injected into the recipient mice, 3×10^6 Min6 cells were recovered by trypsinization (0.05% trypsin/EDTA solution) and suspended in 10 ml of DMEM medium with 15% FCS. The cell suspension was centrifuged at 200 g for 5 min at 4°C. The pellet was suspended in 0.5–0.7 ml of DMEM supplemented with 1 mg/ml nafamostat mesilate (Torii, Tokyo, Japan) and 15% FCS. The cell suspension was kept on ice before the injection.

Transplantation

Transplantation was performed four weeks after STZ injection. 0.4–1.2 U of NPH human insulin (Lilly, Kobe, Japan) was injected subcutaneously in the diabetic mice before the operation to prevent hyperglycemia. Two hundred µl of 10 mg/ml sodium pentobarbital (Abbott, Abbott Park, IL, USA) was injected into the peritoneal cavity to anesthetize the subjects.

The TCPHI method (7) is shown in Fig. 1. The gall bladder is exposed via midline incision. The bottom of the gall bladder is cut with an 18-gauge needle. The tip of a small catheter (1–2 µl GELoader Tips, Eppendorf, Hamburg, Germany), which is connected to a 0.1 mm silicon tube, is inserted into the cholecystic duct. The cell suspension or medium alone (mock transplantation) is injected via the silicon tube. One ml of 20% glucose, 5 mM KCl solution is injected into the duodenum. This procedure is necessary to prevent hypoglycemia during and after

![Fig. 1. The TCPHI method](image)

The cell suspension was injected via gall bladder. The flow to each organ is indicated. (X): the site clamped by forceps. (o): Min6 cells
operation. The gall bladder is then injected with 0.3–0.7 ml of 3×10⁶ cells suspended in DMEM medium supplemented with 1 mg/ml nafamostat mesilate, while the papilla vater is clamped by forceps to block the flow. After the injection is completed, the gall bladder is ligated. Transplantation into the liver is performed by direct injection of 3×10⁶ Min6 cells into the portal vein. 0.4–1.2 U of NPH human insulin (Lilly, Kobe, Japan) is injected subcutaneously for several days after the operation. The levels of blood glucose are measured just before injection of insulin. All of the above experiments were done in accordance with the guidelines of Osaka University.

Analysis of recipient mice

The mice transplanted with Min6 cell line were weighed every week. Nonfasting blood glucose was monitored every two to three days. The blood samples were obtained from the tail vein. Blood glucose was measured using a portable glucose meter (MEDISAFE, Terumo, Tokyo, Japan). When the blood glucose became stable after the transplantation (3 weeks after transplantation), intraperitoneal glucose tolerance test (IPGTT) was performed. After a 17–20 hr fast, the mice were injected intraperitoneally with a 2 g/kg glucose solution. Blood glucose was determined at the time of injection and at 15, 30, 60, 90 and 120 min later. At 0, 15 min, 50 μl of the blood sample was also taken from the tail vein for plasma insulin and immediately mixed with a small volume of EDTA powder to prevent coagulation. Plasma was separated by centrifugation and stored at −80°C until analysis. Plasma insulin levels were determined using EIA mouse insulin detection kit (Morinaga, Yokohama, Japan). Results are expressed statistically as means ± standard error.

Histological examinations

At the end of the experiments, mice were anesthetized (sodium pentobarbital 400 mg/kg ip) and total body, liver and pancreas were weighed. The removed liver and pancreas were fixed in 10% formaldehyde at 4°C for two days and processed for paraffin histology. Deparaffinized sections were subsequently stained with hematoxylin-eosin or processed for immunostaining. Monoclonal anti-insulin antibody (Sigma, St. Louis, MO, USA) was diluted 1:100 in PBS containing 0.05% Tween20 and 1% bovine serum albumin. Sections were incubated for 1 h with primary antibody at 37°C, washed, and covered with secondary Cy5-conjugated anti-mouse antibody (Jackson Immuno Research, West Grove, PA, USA) diluted 1:100 for 1 h at 37°C. The nuclei of each slice were stained with Hoechst 33342 (Sigma, St. Louis, MO, USA). The sections were immersed in mounting medium and covered with coverslips. The samples were subsequently analyzed with a Zeiss LSM410. For hematoxylin-eosin staining, the samples were analyzed with an Olympus PROVIS AX-80.

Statistical analysis

Results were described as means ± standard error. Student’s t-test was used to compare the two groups. Differences were considered significant at a p-value less than 0.05.

Results

STZ-treated diabetic mice recovered insulin secretion

3×10⁶ Min6 cells were transplanted into the diabetic C57BL/6 Tg14 (act-EGFP) OsbY01 mice by the TCPHI method (Fig. 1). Within ten days, nonfasting blood glucose levels fell to the normal range and decreased slowly thereafter (Fig. 2A). On the other hand, the control mice that were mock transplanted showed hyperglycemia during this period (Fig. 2C). This result shows that TCPHI method did not promote pancreatic regeneration, and that the normalization of blood glucose in the Min6 transplanted mice was due to secreted insulin from the transplanted cells. Next we compared the transplantation efficiency between the intraportal infusion and TCPHI method. Min6 cells were injected from the portal vein and directly transplanted into the liver. The time courses of blood glucose are shown in Fig. 2B. Mice transplanted with 3×10⁶ Min6 cells via the portal vein showed no significant difference compared with the TCPHI method (p<0.05) (Fig. 2D). These results show that the Min6 cells were effectively transplanted in the pancreas and liver by the TCPHI method, and that the efficiency was almost the same as the intraportal injection method.
Fig. 2. Changes in blood glucose concentrations after transplantation of Min6 cells into hyperglycemic mice
The diabetic mice were transplanted 3 × 10^6 Min6 cells (A, white squares) or mock-transplanted (C, white triangles) by the TCPHI method (n = 9). The diabetic mice were transplanted 3 × 10^6 Min6 cells by intraperitoneal injection method (B, black circles) (n = 9). Mean values are shown ± SE. (D) The mean values of A, B and C are shown in the same field. The vertical line shows the concentration of blood glucose (mM). Blood glucose levels more than 33.3 mM were out of range of the portable glucose meter. The horizontal line indicates the number of days after transplantation. The transplantation was performed at day 0.

Transplantation prevented hyperglycemic cachexia

Body weight of normal controls and the Min6 transplanted mice are shown in Fig. 3. Diabetic mice lost body weight (~ 4 g) within three weeks after STZ administration. Transplantation of Min6 cells into STZ diabetic mice resulted in restoration of normoglycemia within 10 days. Water intake and urine volume decreased progressively within one week. The body weight of diabetic mice increased rapidly within three weeks following transplantation and reached a plateau at a level (24 g) that was lower than that of normal controls.

Transplanted Min6 cells responded to intraperitoneal glucose loading

After blood glucose levels plateaued, intraperitoneal glucose tolerance test was performed. The

Fig. 3. Recovery of body weight loss due to hyperglycemia after implantation of Min6 cells into the pancreas and liver
Twelve week-old normal controls (white squares) and the streptozotocin treated diabetic mice (black circles) were weighed every week (n = 9). At day 0, Min6 cells were transplanted into diabetic mice. Mean values are shown ± SE.
The number of transplanted Min6 cells was equivalent to 120 islets and responded to glucose loading in the pancreas and liver. Basal glucose concentration of the transplanted mice was significantly (p = 0.006) lower than that of the normal controls after an overnight fast (Fig. 4A). This is because basal insulin secretion was not inhibited completely at low blood glucose levels in Min6 transplanted mice. Blood glucose levels of Min6 transplanted mice were lower than that of the normal controls (Fig. 4A). Insulin level at 15 min of the Min6 transplanted mice was four times higher than that of the normal controls (p = 0.002) (Fig. 4B). Serum insulin levels at 15 min showed a 4.6-fold increase of the Min6 transplanted mice compared with the 2.3-fold increase in normal control (Fig. 4B).

**Min6 cells settled down near the ducts**

The liver and pancreas sections were stained with hematoxylin-eosin or immunostained with anti-insulin antibody (Fig. 5). Min6 cells were detected readily throughout the liver and pancreas. Min6 cells often occurred as loose aggregates in the plane of liver section (Fig. 5A). Cell clusters obstructing the pancreatic or bile ducts were observed coming out of the ducts where they had settled. All the Min6 cells had reduced cytoplasmic volumes and small, hyperchromatic nuclei. In the liver, Min6 cells showed the islet-like structure that was insulin positive (Fig. 5B). As injected Min6 cells were labeled with SP-Dil, the cell clusters in the pancreas were detected under fluorescence (Fig. 5C). Some of these clusters resembled intrinsic islets. When the aggregates were stained with anti-insulin antibody, the SP-Dil and insulin double positive Min6 cells (Fig. 6D) were distinguished from SP-Dil negative and insulin positive intrinsic islets. The cell clusters located near the pancreatic ducts (Fig. 5C, D) and some clusters proliferated in the duct wall (data not shown). All these data show that Min6 cells were transplanted near the peripheral ducts of pancreas and liver.

**Transplanted Min6 cells did not affect organ weight**

The weight of pancreas and liver were measured at the end of the experiments. As the cells were transplanted into these organs, the high concentration of insulin may have affected the local environment. There was a report that acinar cells around the islets
showed the periinsular halos in which the acinar cells were large [12]. The weight of the liver and pancreas were compared with that of the normal control mice (Table 1), but the weights of both organs were not significantly different between normal controls and transplanted mice (p>0.05). These results show that Min6 cells did not affect the weight of the liver or pancreas in the 40 day experimental period. Microscopically, there was no difference in the shape and size of the acinar cells and hepatic cells around the Min6 cell clusters (Fig. 5B, C).

Discussion

A Min6 cell suspension was injected into the choledochal duct, allowing the cells to flow into the pancreas and liver. If the common bile duct was clamped, we could inject the solution selectively into the liver forcing the cells to pass through the common hepatic duct to transplant themselves in the liver. On the contrary, selective transplantation into the pancreas was quite difficult as the common hepatic duct was too small to stop the flow by forceps. In all our experiments, the cells were transplanted into both the pancreas and liver.

As the operational stress was fairly severe, all the mice without insulin therapy died within a day. Insulin therapy prevents the negative effects of hyperglycemia on islet revascularization [13, 14]. Transplanted Min6 cells also have an insulin response to the recipient hyperglycemia. The injected and Min6-secreted insulin both induced severe transient hypoglycemia. A 20% glucose, 5 mM KCl solution had to be introduced into the duodenum before cell injection to prevent hypoglycemia.

The injection volume can be increased up to 1 ml, while the pancreas is still intact. We carried out transplantation in more than one hundred mice but operational death was detected in only 5% of the transplanted cases. Most of the successful cases did not show severe pancreatitis. We speculate that the intraductal injection of enzyme inhibitor might have exerted a protective effect on the pancreas. Cell transplantation can be done without any serious problems in the TCPH assay.

Min6-cell line derived from insulinoma of the transgenic C57BL/6J mouse. The genome contains the SV40-T antigen gene under the control of the human insulin promoter. This cell line expresses the SV40-T antigen that might trigger a host immune response. But Min6 cells were successfully transplanted in db/db mice that derived from C57BL/KsJ [15]. Our results also proved that Min6 cells could be transplanted into C57BL/6J mice without rejection.

Nonfasting blood glucose levels fell to the normal range within 10 days after transplantation and decreased slowly thereafter. Finally, all the mice died.
within two months due to severe hypoglycemia. Min6 cells were also injected subcutaneously but the result was the same (data not shown). The transplanted Min6 cells started to secrete more insulin independent of the injected site. Our results are different from the previously reported case [15]. In db/db mice, blood glucose levels remained unchanged for five months. As Min6 cell line originated from insulinoma, the basal insulin secretion was not completely inhibited below normal level. The db/db mice in the previous reported case may have lived as a result of the equilibrium established between the gain of insulin resistance [16] and the increase of insulin secretion. Continuous increase of body weight after transplantation induced glucose intolerance. Another explanation is that the Min6 cells with a different characteristic might be predominant in our experiment. Min6 cells grew very slowly and three months were necessary to obtain sufficient cell numbers to study their function. During this period, fast growing cells with high basal insulin secretion may have become predominant.

The insulin content of an islet cell is five times more than that of a Min6 cell [10]. The mean number of beta cells per rat islet is from 3640 ± 620 to 12815 ± 710 [17]. A human islet has about 4000 beta cells per islet [18], and there is little difference in the number of beta cells per islet among other species. In mouse syngenic islet transplantation, at least 50 islets are necessary for complete reversion of hyperglycemia [19]. We calculated 50 islets × 5000 beta cells × 5 times = 1.25 × 10⁶ Min 6 cells. This equation means that 50 islets are equivalent to 1.25 × 10⁶ Min 6 cells. In this experiment, we transplanted 3 × 10⁶ Min6 cells into the pancreas and liver. Theoretically, this cell mass is equal to 120 islets and equivalent to 4800 islet equivalent/kg. Intrapancreatic and hepatic implantation of Min6 cells into STZ-diabetic mice resulted in prompt restoration of normoglycemia, and all the mice showed a blood glucose level between 5.5–11 mM in two weeks (Fig. 2). In IPGTT, fasting plasma glucose levels of Min6 transplanted mice were lower than that of the normal controls (Fig. 4A). As intraperitoneal glucose tolerance test was performed three weeks after transplantation, blood glucose levels of the transplanted mice were lower than that of the control.

There were several problems in transplanting the cells using the TCPHI method. First, as the pancreas and liver secrete proteinase, lipase or bile acid, respectively, which are toxic to the cells, there was the possibility that these toxic substances might destroy most of the injected cells. This problem was solved by the addition of enzyme inhibitor and dilution with injected medium. Next the efficiency of transplantation was dependent on cell settlement. While the injected cells flowed upstream in the ducts, the hydrostatic pressure of both the injected solution and the secreted digestive juices pushed some of the cells back. The injected cells needed to settle down in the ducts. After the cell suspension was injected, the pancreas enlarged initially but gradually decreased in size during the operation. Although we could not control the cell suspension leaking from the papilla vater, by using the TCPHI method, we did not need to repeat the injection despite the fact that all the procedures were performed only once [20, 21]. As a result, the transplanted Min6 cells normalized hyperglycemia. The histological analyses of Min6 transplanted organs indicated that most of the cells formed clusters near the small ducts. The injected cells were believed to have caused obstruction in the ducts. The cells were released from the ducts and formed those clusters around the duct. The obstruction presumably occurred within a short time (about 5 min) after the injection. Interpretation of these results, based on the estimation of the injected cell number indicated in the previous paragraph, the transplantation efficiency of this step was enough to normalize blood glucose level. The third issue we had to face was vascularization. In Fig. 2, the graph of blood glucose levels showed three distinct phases. Prior to the operation, blood glucose levels were between 22 mM and 32 mM. Within the first 10 days following the operation, the rapid decline in blood glucose levels correlated with vascularization. The duration of this vascularization period was similar to that of other reported cases [13, 22].

Pancreatic and hepatic cell lineage have long been a matter of speculation. Gene manipulation technology has elucidated many important factors in their development [23]. Tissue regeneration shares some common factors with organ development, but direct differentiation assay is necessary to confirm their function [24, 25]. We devised an in vivo system to analyze transplanted cell activity in the functional regeneration of the pancreas. Both in the pancreas and the liver, the ducts drain digestive liquids into the duodenum. As acinar [26] or hepatic cells became
atrophic after duct ligation [27], stem cells need to differentiate into the pancreas or liver cells near the ducts. At the same time, the ducts harbor intrinsic stem cells. While no clearly designated niche seems to exist around the duct tubes [28], there is clearly a supportive system for stem cell differentiation around the pancreatic ducts [3, 25]. Our method fully utilized the dense network of ducts to transplant cells adjacent to the differentiation machinery in the ducts. Long-term failure of the intraperitoneally transplanted islets was due to the number of islet cells [21, 29–31]. The loss of function in these cases may also have been the result of the ectopic location of islets.

It is likely that the survival rate of functional islets may be enhanced under the condition that islets are supplied by stem cells [32]. The TCPHI method could reconstruct the supportive machinery in the pancreas or liver.

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