Effects of encapsulated porcine islets on glucose and C-peptide concentrations in diabetic nude mice 6 months after intraperitoneal transplantation

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Abstract

**Background:** In patients with type 1 diabetes, allogeneic islet transplantation can provide normal HbA1c concentrations, but it requires immunosuppression. Transplanting encapsulated islets into the peritoneal cavity could reduce or eliminate the need for immunosuppression. One of the uncertain features of intraperitoneal islet transplantation is the difficulty of measuring C-peptide concentrations in peripheral blood, which is often used for the marker of islet function. We hypothesized that secreted C-peptide from intraperitoneally transplanted islets was mostly consumed in the peritoneal cavity, which resulted in low C-peptide concentrations in peripheral blood.

**Methods:** In each of two experiments, encapsulated neonatal porcine islets were intraperitoneally transplanted into four nude mice with streptozotocin-induced diabetes. Three diabetic nude mice without transplanted islets were used as diabetic controls, and three untreated healthy nude mice were used as normal controls. Islet functions were monitored for 2 months in the first experiment and 6 months in the second experiment. Encapsulated islets were retrieved after each experiment and evaluated by fluorescein diacetate/propidium iodide tests for the viability and static glucose-stimulated insulin release tests for the function. C-peptide concentrations from the blood and from the intraperitoneal cavity at 6 months were compared.

**Results:** In both experiments, diabetes was reversed in all transplanted mice, and oral glucose tolerance test showed improved profiles. In general, retrieved islets were viable and functional. However, blood porcine C-peptide concentrations were low at both 2 and 6 months, and concentrations in the ascites of peritoneal cavity were 40 times as high as those in blood.

**Conclusions:** The peripheral blood sampling for c-peptide, though highly informative in vascularized grafts, may not be the primary tool for monitoring the health and function of encapsulated products when transplanted into intraperitoneal cavity. Our
1 | INTRODUCTION

Intensive insulin therapy, the standard treatment for type 1 diabetes, can reduce HbA1c concentrations, but it also increases the number of hypoglycemic episodes. Although both allogeneic pancreas and islet transplantation can provide normal HbA1c concentrations without increasing hypoglycemic episodes, transplantation requires immunosuppressive therapy. Encapsulating islets could reduce or eliminate the need for immunosuppression, but because encapsulation increases the size of the islets, they were usually transplanted into the peritoneal cavity instead of into the standard hepatic sites. Several clinical trials have transplanted both encapsulated human and porcine islets into the peritoneal cavity. One of the uncertain features of intraperitoneal encapsulated islet transplantation is the difficulty of measuring C-peptide concentrations in peripheral blood. C-peptide concentrations are often used for the marker of function of transplanted islets; therefore, difficulty of measuring C-peptide concentration might be confused with the non-function of islets.

In healthy people, insulin secreted by the pancreas is transported to the liver, where between 30% and 80% is metabolized. Therefore, intraperitoneal injections of insulin are considered to be more physiologic than subcutaneous injections because insulin is absorbed by the capillaries of the visceral peritoneum into the portal vein. In fact, intraperitoneal insulin injection was shown to be more beneficial than standard the subcutaneous insulin injection. At the same time, injected intraperitoneal insulin results in high hepatic insulin uptake and relatively low peripheral insulin concentrations.

Considering together, we hypothesized that intraperitoneally transplanted islet cells would provide high concentrations of insulin and C-peptide in the peritoneal cavity, that, when metabolized, would reduce concentrations of insulin and C-peptide in the peripheral blood.

In this study, we analyzed C-peptide concentrations in the peritoneal cavity and peripheral blood on the diabetic nude mice after intraperitoneal encapsulated porcine islet transplantation. C-peptide concentrations in the peripheral blood were much lower than the intraperitoneal concentrations in the transplanted nude mice. Our results may explain why intraperitoneal encapsulated islet transplantation in humans is associated with limited C-peptide concentrations in the peripheral blood.

2 | MATERIALS AND METHODS

The study was approved by the Committee on the Care and Use of Laboratory Animals of Otsuka Pharmaceutical Factory, Inc.

2.1 | Materials

ET-Kyoto solution was supplied by Otsuka Pharmaceutical Factory, Inc. (Naruto, Japan). All other reagents were of commercially available analytical grade.

2.2 | Animals

Newborn piglets were provided from Kadoi Ltd (Ibaraki, Japan). The piglets were brought to Haneda airport by a car. Then, they were brought to Osaka airport by air and then brought to Kobe (TransGenic Inc., Kobe, Japan) by a car. Six newborn piglets (11 days old) were used at experiment 1, and 12 newborn piglets (12 days old) were used at experiment 2.

Male nude mice between 7 and 8 weeks old (BALB/cA Jcl-nu/nu) were provided by CLEA Japan, Inc. (Tokyo, Japan). Nude mice were housed in a light-controlled room (12:12-hour light-dark cycle, lights off at 19:00). Average temperature was kept at 23±3°C (mean±Standard Deviation; SD), and humidity was kept at 55±15%. Mice were allowed free access to water and were fed a standard rat diet containing (per 100 g body weight) 54.5 g of starch, 22.4 g of protein, 5.7 g of fat, 3.1 g of fiber, and 6.6 g of mineral and vitamin mixture (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan). Nude mice before and after transplantation were reared in grouped and in isolated, respectively.

2.3 | Preparation, culture, and characterization of encapsulated islets

Six piglets (experiment 1) and 12 piglets (experiment 2) were euthanized, and their pancreases were procured with sterile technique at the TransGenic Inc. Pancreata were preserved with ice-cold ET-Kyoto solution and shipped to Otsuka Pharmaceutical Factory, Inc. (Naruto, Japan). Cold preservation time was approximately 3 hours.

All procured pancreata from 6 piglets (experiment 1) and 12 piglets (experiment 2) were processed to isolate islets as previously described. Isolated neonatal porcine islets were then encapsulated at Otsuka Pharmaceutical Factory Inc. with alginate poly-L-ornithine-alginate (APA) as previously described. Briefly, the alginate encapsulated islets were coated with two different concentrations of PLO. Then, the core of alginate was liquefied using citrate buffer. This method created a capsule consisted of single layer of PLO-Alginate complex. The encapsulated islets were cultured for an additional 3 weeks for maturation. Islet yield was determined using dithizone staining (Sigma Chemical Co., St. Louis, MO, USA) (2 mg/mL) under optical reticule and converted into a standard number of islet equivalents (IEQ, diameter standardizing to 150 μm). In addition, purity
The function of APA-encapsulated neonatal porcine islets was assessed in vivo. To induce diabetes, 240 mg/kg body weight streptozotocin was injected into the abdominal cavities of seven healthy nude mice. None of mice were dead after streptozotocin injection. Glucose concentrations were measured from blood samples taken from the tail vein. Non-fasting blood glucose concentrations were measured using a LIFE CHECK (Gunze Ltd, Kyoto, Japan). A non-fasting blood glucose concentration greater than 300 mg/dL was defined as diabetes. The duration of diabetes was less than 1 week before transplantation. After transplantation, a non-fasting blood glucose concentration less than 200 mg/dL on 2 or more consecutive measurements was defined as reversal of the diabetes.

Encapsulated neonatal porcine islets (4000 IEQ) were transplanted into four of the seven streptozotocin-induced diabetic nude mice. All four transplantations were successful, and none of transplanted mice were dead. As diabetic controls, no islets were transplanted into the remaining three streptozotocin-diabetic nude mice. In addition, three untreated, healthy nude mice were assessed as normal controls. We selected n=4 for the initial pilot study in experiment 1. Then, we hypothesized there should be huge differences of C-peptide concentration between abdominal fluid and peripheral blood. Therefore, we selected n=4 to achieve significant differences in experiment 2. We selected n=3 for both healthy and diabetic mice, because healthy control provides less variable data and diabetic mice suffered serious ill and dead within 2 weeks constantly.

Oral glucose tolerance tests were conducted on days 13, 32, and 61 after transplantation with all seven surviving mice (four transplanted and three healthy controls; the diabetic controls were euthanized due to the adverse effects of high concentrations of glucose). All mice were fasted for overnight (more than 16 hours) before OGTT. Blood samples from the tail vein were collected for measuring blood glucose concentration before and 0, 5, 15, 30, 60, 90, and 120 minutes after an oral injection of dextrose 1 g/kg body weight.

On day 62, the four transplanted mice and three healthy controls were euthanized under general anesthesia using isoflurane. Porcine C-peptide concentrations were measured from blood collected from the heart. Samples were centrifuged at 3000 g at 4°C for 10 minutes and the plasma collected. Plasma porcine C-peptide concentrations were measured using the Mercodia Porcine C-peptide ELISA kit (Mercodia, Sweden) according to the manufacturer’s instructions. The transplanted encapsulated islets were retrieved from the abdominal cavities. For the retrieval of encapsulated islets, laparotomy was performed using midline incision. Then, entire abdominal cavity was washed with Hank’s balanced salt solution several times and all washed materials were collected into 35-mm dishes. Encapsulated islets were detected under a microscope.

### 2.4 Experimental protocols

#### 2.4.1 Experiment 1: 2-month assessment of in vivo islet function

To observe the long-term efficacy of encapsulated islets, the second experiments were conducted. Encapsulated neonatal porcine islets (4000 IEQ) were transplanted into four diabetic nude mice to assess the encapsulated islets for up to 6 months. All four transplantations were successful, and none of transplanted mice were dead. Non-fasting blood glucose concentrations were measured. For this set of experiments, oral glucose stimulation tests were not conducted during follow-up period because they are too stressful in small diabetic nude mice, which might hamper the long-term survival.

Between 182 and 187 days (about 6 months) after transplantation, four transplanted mice were euthanized under general anesthesia using isoflurane. Porcine insulin, C-peptide, and glucose concentrations were measured in blood collected from the heart. Plasma was collected after centrifugation, as described above. Porcine insulin, C-peptide, and glucose concentrations were also measured in ascitic fluid. Plasma and ascitic fluid glucose were measured using an automatic analyzer (Model 7180; Hitachi High-Technologies Corporation, Tokyo, Japan). Plasma and ascitic fluid porcine insulin and C-peptides were measured using Mercodia Porcine insulin and C-peptide ELISA kits (Mercodia, Sweden), respectively, according to the manufacturer’s instructions.

The transplanted encapsulated islets were then retrieved from the abdominal cavity as described above.

#### 2.4.2 Experiment 2: 6-month assessment of encapsulated islets

In the retrieved encapsulated islets, recovery rate was determined as the recovered IEQ divided by the transplanted IEQ, viability was determined by FDA/PI staining, and purity was determined by the dithizone-positive rates.
2.6 Statistical methods

Data are reported as means and standard deviations (mean±SD). Outcomes in the transplanted and positive-control groups were compared with unpaired t-tests. Alpha was set at 0.05, and all tests were two-tailed. All data were analyzed with Microsoft Excel 2010.

3 RESULTS

3.1 Islet characterization

Before transplantation, the viabilities of encapsulated islets were 84.0% and 94.5% in experiments 1 and 2, respectively (Table 1). The dithizone-positive rates were 65.7% and 67.5% in experiments 1 and 2, respectively (Table 1). Insulin levels during SGS assays were 7.2, 50.5, and 14.6 (μU/100 IEQ/h) in L1, H, and L2 in experiment 1 and 15.3, 96.6, and 27.4 (μU/100 IEQ/h) in experiment 2, respectively (Figure 1A). S1 and S2 were 7.3 and 3.5 in experiment 1 and 6.1 and 3.8 in experiment 2, respectively (Figure 1B).

3.2 In vivo function test

In experiment 1, due to the aggravation of condition with high blood glucose, diabetic nude mice without transplantation were sacrificed after day 5. The blood glucose levels of diabetic nude mice with transplantation were progressively approaching to normal healthy levels (Figure 2A) with increasing body weight (Figure 2B).

Oral glucose tolerance tests were conducted with surviving mice: all four transplanted mice, and all three normal healthy control mice. Transplanted mice showed improved glucose metabolic profile at 2 weeks, 1 month, and 2 months (Figure 3). In fact, transplanted mice showed lower blood glucose than normal healthy mice in all periods.

In experiment 2, for long-term assessment, non-fasting blood glucose levels after transplantation were also measured up to 6 months. The non-fasting blood glucose levels were also progressively approaching to normal healthy levels (Figure 4A) with increasing body weight (Figure 4B).

| TABLE 1 Characterization of APA-encapsulated islet before intraperitoneal transplantation at experiment 1 and 2 |
|--------------------------------------------------------------------------------------------------|
| experiment 1 | experiment 2 |
|----------------------------------|----------------|
| Viability (%) | 84.0 | 94.5 |
| Dithizone positive rate (%) | 65.7 | 67.5 |

**FIGURE 1** (A) Insulin release during glucose-stimulated insulin secretion tests on APA-encapsulated islet cells before intraperitoneal transplantation at experiments 1 and 2, (B) its stimulation indices (SI), (C) Insulin release during glucose-stimulated insulin secretion tests on retrieved APA-encapsulated islet cells, and (D) its stimulation indices (SI). Tests were performed in triplicate. Values at (A) and (B) represent single (n=1), and values at (C) and (D) represent means ± SD (n=4). Insulin concentrations in supernatants were measured with porcine insulin ELISA kits (Mercodia, Uppsala, Sweden). L1, H, and L2 are the insulin concentration at the initial low glucose, high glucose, and the second low glucose concentrations, respectively. *

* S1 was significantly higher at 6 mo than at 2 months.
3.3 | Assessment of the porcine C-peptide levels and retrieved encapsulated islets

The transplant group (n=4), peripheral blood showed positive porcine C-peptide, with a value of 71±18 pmol/L at day 62 (Figure 5). All healthy mice showed negative porcine C-peptide concentration. Porcine C-peptide concentrations were also positive at 6 months, with a value of 94±17 pmol/L. There was no significant difference of the values of porcine C-peptides between at day 62 and 6 months (P=.11) (Figure 5).

Retrieved islets were compared with the recovery rates, viability, and purity between the 2-month and 6-month groups (Table 2). Six-month group showed significant better outcomes compared with the 2-month group in viabilities although viabilities were very high in both groups. There were no significant differences in recovery rate and dithizone-positive rates between the 2-month and 6-month groups (Table 2).

SGS outcomes for retrieved encapsulated islets were compared between the 2-month and 6-month groups. In both groups, insulin levels were increased by high glucose stimulation and returned to low by low glucose environments. There were no significant differences in insulin levels at L1, H, and L2 (Figure 1C). However, SI1 was significantly higher in 6-month group (11.9±4.4 vs 24.9±2.8, in 2 months and 6 months, respectively, P<.01), and SI2 was also higher in 6-month group (2.4±0.6 vs 3.7±1.0 in 2 months and 6 months, respectively, P=.07) even there was no significant difference (Figure 1D). Of note, SI1 values were increased after transplantation compared with pre-transplantation values.

In general, retrieved encapsulated porcine islets were viable and functional at both 2 months and 6 months. In fact, viability and SI1 were significantly improved at 6 months.

Representative images of both dithizone and FDA/PI staining of encapsulated porcine islets before (A and C, respectively) and after (B and D) transplantation at 6 months were shown (Figure 6). In dithizone staining, red staining indicates insulin-positive cells. In FDA/PI staining, red staining indicates dead cells, and green staining indicates live cells. Of note, small portion of capsules were fibrotic and broken (Figure 6B, allow).

3.4 | Comparison of plasma and ascitic fluid levels of glucose, porcine insulin, and C-peptide

After 6 months of transplantation, we compared plasma and ascitic fluid levels of glucose concentrations, insulin, and porcine C-peptide
concentrations (Table 3). Glucose concentrations were similar between two groups. However, porcine insulin concentrations were approximately 400 times and C-peptide concentrations were approximately 40 times higher in the ascitic fluids compared with the levels in plasma (peripheral blood).

4 | DISCUSSION

After intraperitoneal transplantation of encapsulated islets, C-peptide concentrations in peripheral blood are usually low and sometimes undetectable even apparent efficacy of islets were present. As C-peptide concentration is often used as the marker for the islet function, difficulty of measuring the C-peptide concentration might confuse with the non-function of islets.

We hypothesized that intraperitoneally transplanted encapsulated islets would secrete insulin into the peritoneal cavity, where it would be used locally, and where only a limited amount would enter the peripheral blood stream.

FIGURE 3 Blood glucose profiles oral glucose tolerance tests in normal healthy mice (solid circles; n=3) and transplanted diabetic nude mice with transplanted (open circles; n=4) at (A) 2 wks (13 d), (B) 1 mo (32 d), and (C) 2 mo (61 d) after transplantation. Results are shown as mean±SD.

FIGURE 4 (A) Non-fasting blood glucose concentrations and (B) body weights in four transplanted diabetic nude mice.

FIGURE 5 Porcine C-peptide concentrations in the plasma in 2 mo (62 d) and 6 mo (182-187 d) after intraperitoneal transplantation of APA-encapsulated islet. Values represent means±SD (n=4).

In the first experiment, 4000 IEQ of encapsulated neonatal porcine islets transplanted into the peritoneal cavity reversed streptozotocin-induced diabetes for up to 2 months. Non-fasting blood glucose concentrations reduced toward normal levels, and oral glucose tolerance tests demonstrated that islets responded to glucose. In fact, during oral
TABLE 2  Characterization of retrieved APA-encapsulated islet at 2 mo (62 d) and 6 mo (182-187 d) after intraperitoneal transplantation

|                  | 2 mo       | 6 mo       | P  |
|------------------|------------|------------|----|
| Recovery rate (%)| 92.1±7.4   | 76.0±15.4  | .11|
| Viability (%)    | 95.8±0.6   | 100.0±0.0  | <.001|
| Dithizone positive rate (%) | 95.3±1.4 | 90.0±8.5  | .27|

Values represent means ± SD (n=4 in both groups).

After oral glucose tolerance testing, blood glucose concentrations were lower in the transplanted mice than they were in the healthy controls. On the other hand, mean plasma porcine C-peptide concentration in transplanted mice was only 71 pmol/L (approximately 0.2 ng/mL) at 2 months. Considering the well-reduced non-fasting blood glucose concentrations and the glucose profiles during oral glucose tolerance testing, we speculated that C-peptide concentrations in the peritoneal cavity might be higher than that in the peripheral blood. We also speculated that encapsulated porcine islets could survive a long time, even in mice. We noticed that non-fasting blood glucose concentrations temporarily increased after oral glucose tolerance test, so we did not include this test in the second set of experiments with longer follow-up times.

In the second set of experiments, a new set of transplanted mice were followed for up to 6 months. In addition, encapsulated islets were retrieved from the peritoneal cavity, and glucose concentration, porcine insulin, and C-peptide concentrations in the peripheral blood and ascitic fluids were measured simultaneously at 6 months. As we speculated, the four transplanted mice survived well and maintained well reduced non-fasting blood glucose concentrations for up to 6 months. As we did not measure mouse C-peptide levels, it might be possible to regenerate original beta cells in mice. However, as our retrieved encapsulated islets secreted more insulin than the islets before transplantation did, it should be reasonable to consider that the transplanted islets functioned for maintaining improved glucose levels.

On the other hand, we performed SGS assay with 100 IEQ to 500 IEQ encapsulated islets, which could be considered variable. However, as we used the same islets for each assay, stimulation index could be more reliable than L1, H, L2 values. Of note, we have created a standard operative procedure for SGS assay; therefore, this assay becomes reliable now.

Mean porcine C-peptide concentrations in peripheral blood even slightly increased, to 94 pmol/L, at 6 months. At 6 months after

FIGURE 6  Dithizone and FDA/PI staining of encapsulated porcine islets before transplantation (A and C, respectively) and after transplantation and retrieval from diabetic nude mice (B and D, respectively). In dithizone staining, red indicates insulin-positive cells. In FDA/PI staining, red indicates dead cells, and green indicates living cells. The arrow at B) indicates the fibril formation

TABLE 3  Glucose and porcine C-peptide levels in the plasma and ascitic fluid at 6 mo (182-187 d) after intraperitoneal transplantation of APA-encapsulated islet

|                          | Plasma   | Ascitic fluid | P    |
|--------------------------|----------|---------------|------|
| Glucose level (mg/dL)    | 120±32   | 112±28        | .71  |
| Porcine insulin level (μg/L) | 0.30±0.15 | 122±74        | <.02 |
| Porcine C-peptide level (pmol/L) | 94±17 | 3931±1521     | <.01 |

Values represent means ± SD (n=4).
transplantation, mean intraperitoneal porcine insulin and C-peptide concentrations were very high compared to the value in the peripheral blood. The low blood glucose concentrations found during oral glucose tolerance test could be associated with this high concentration of porcine insulin and C-peptide in the peritoneal cavity. Gazda et al. also reported that porcine C-peptide was present in the peritoneal fluid of transplanted dogs at approximately 3 months, but serum porcine C-peptide was not detected at this time.18

Blood glucose concentrations in the peripheral blood and peritoneal ascitic fluid were also similar. This similarity supports the possibility that high insulin concentrations in the peritoneal cavity allow insulin to enter the liver through the peritoneum, where it is adequately metabolized and produces improved blood glucose concentrations. Glucose concentrations can then rapidly equilibrate through the whole body.

However, what happens to C-peptides as they move from the peritoneal cavity to the peripheral blood is unclear. Measuring C-peptide concentrations in portal and peripheral blood could address this question. In fact, we are currently conducting this study at our laboratory.

In diabetic nude mice, the transplanted encapsulated islets survived well and secreted sufficient amounts of insulin for up to 6 months. The secreted insulin could be used in the abdominal cavity, and only limited amounts would enter the peripheral blood. Even at low concentrations of porcine C-peptide, non-fasting blood glucose concentrations in the peripheral blood were well reduced, most likely because the amount of insulin in the peritoneal cavity was sufficient.

The transplanted mass of 4000 IEQ/mouse is approximately 200,000 IEQ/kg, which is 10-20 times higher than that in humans. In this study, we retrieved 92.1% and 76% of encapsulated islets from the peritoneal cavity at 2 and 6 months, respectively. Even with such high proportions of surviving islets, porcine C-peptide concentrations in the peripheral blood were low enough to be barely measurable. Therefore, at standard doses of transplanted encapsulated islets, detecting porcine C-peptide in the peripheral blood might be difficult. Of note, this high amount of transplanted islets might be a reason for relatively low blood glucose concentrations during the OGTT.

In this study, we used athymic nude mice to minimize the effect of immune responses. The transplant results might have been different in the case of immune-competent mice, in particular the quality of the microcapsules and their cellular component after several months.

In conclusion, we found that encapsulated neonatal porcine islets transplanted into the intraperitoneal cavity could reverse diabetes in nude mice and maintain normoglycemia for up to 6 months. Porcine C-peptide concentrations were high in the peritoneal cavity and low in the peripheral blood, which could explain why in clinical practice, transplanted encapsulated islets are associated with limited C-peptide concentrations in the circulating peripheral blood.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

Masuhiro Nishimura, Naho Iizuka, Yasutaka Fujita, Osamu Sawamoto, and Shinichi Matsumoto involved in the concept and design of the study; performed the data collection, analysis, and interpretation; drafted the manuscript; and involved in the critical revision and approval of the article.

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