

## Exendin-4 Treatment Expands Graft $\beta$ -Cell Mass in Diabetic Mice Transplanted With a Marginal Number of Fresh Islets

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Exendin-4 stimulates insulin secretion, suppresses glucagons secretion, increases  $\beta$ -cell replication and neogenesis, and reduces  $\beta$ -cell apoptosis. However, it has been shown that posttransplant exendin-4 treatment did not improve glucose homeostasis in diabetic mice transplanted with a large number of freshly isolated islets. The aim of this study was to test if exendin-4 is beneficial for hyperglycemic recipients with a marginal number of fresh islets. We transplanted 150 C57BL/6 mouse islets under the kidney capsule of inbred streptozotocin-diabetic mice, and then treated the recipients with and without exendin-4 for 6 weeks. Before and after transplantation, recipients' blood glucose, body weight, and intraperitoneal glucose tolerance test were measured. At 6 weeks, the grafts were removed to determine  $\beta$ -cell mass. Blood glucose levels in both groups decreased progressively after transplantation, and the exendin-4-treated group had had lower blood glucose than controls since day 3. By 6 weeks, euglycemia was achieved more in mice treated with exendin-4 than in controls (100% vs. 62.5%,  $p = 0.018$ ). The time to obtain normoglycemia was shorter in the exendin-4-treated group than in controls ( $12 \pm 8$  vs.  $29 \pm 13$  days,  $p < 0.001$ ). Blood glucose at 6 weeks was  $123 \pm 18$  and  $170 \pm 62$  mg/dl in the exendin-4-treated group and controls, respectively ( $p = 0.008$ ). Additionally, the exendin-4-treated group had better glucose tolerance than controls at 2 and 4 weeks ( $p < 0.02$ ). However, both groups exhibited increased body weight over time, and weight changes did not significantly differ between the two groups throughout the study period. At 6 weeks after transplantation, grafts in the exendin-4-treated group were more prominent and contained more insulin-stained cells than those of controls. They had 2.3-fold  $\beta$ -cell mass of the graft compared with controls ( $0.30 \pm 0.11$  vs.  $0.13 \pm 0.03$  mg,  $p = 0.012$ ). These results indicate posttransplant exendin-4 treatment in the diabetic recipient with a marginal number of fresh islets expands graft  $\beta$ -cell mass and improves transplantation outcome.

Key words: Diabetes mellitus; Islet transplantation; Exendin-4;  $\beta$ -Cell mass

### INTRODUCTION

Recently, the Edmonton Protocol has markedly improved the success rate of human islet transplantation (30). However, two or more pancreases are usually required to achieve normoglycemia. Moreover, long-term function of the transplanted islets has been disappointing; only 10% of patients maintain insulin independence 5 years after transplantation (29,31). Allograft failure may be due to nonimmunological (e.g., insufficient  $\beta$ -cell mass and islet engraftment problems) as well as immunological (e.g., immune rejection, toxicity of immunosuppressants, and autoimmune recurrence) factors. To improve the outcome of islet transplantation, these problems have been intensively investigated (28).

The shortage of human donor pancreata has prompted

efforts to expand the human donor pool, modify islet processing and preservation methods, as well as identify alternative islet sources (28). Another important approach is the generation of new  $\beta$ -cells either from preexisting  $\beta$ -cells or from progenitor/stem cells. Recent studies have shown that, even in patients with longstanding type 1 diabetes, the endogenous pancreas maintains the ability to resupply, but new insulin-producing cells to compensate for  $\beta$ -cell mass are lost as a consequence of autoimmune injury (24). The glucagon-like peptide (GLP)-1 improves glycemic control in type 2 diabetic patients by stimulating glucose-dependent insulin secretion and biosynthesis and by suppressing glucagon secretion, gastric emptying, and appetite (8,9). Additionally, GLP-1 is also known to expand  $\beta$ -cell mass by stimulating  $\beta$ -cell proliferation and inhibiting  $\beta$ -cell apoptosis (2,7,23,34).

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However, clinical application of native GLP-1 is limited due to its very short plasma half-life (19).

Exendin-4 (exenatide) is a GLP-1 mimetic resistant to dipeptidyl peptidase-IV-mediated inactivation and thus exhibits more sustainable effects (13). It also has the ability to expand  $\beta$ -cell mass via stimulation of  $\beta$ -cell replication and neogenesis (2,21,23,27,35,36) as well as prevention of  $\beta$ -cell death (2,5,22,33). A recent study demonstrated that, in diabetic mice, syngeneic transplantation of islets precultured with exendin-4 increases the reversal rate of hyperglycemia. In contrast, posttransplant exendin-4 treatment did not improve glucose homeostasis in diabetic recipients initially transplanted with a large number of freshly isolated islets (20). Because transplantation of additional islets can enhance the growth and function of the islet graft (15, 16,26), the beneficial effects of exendin-4 can be masked. Therefore, in the present study, we tested if posttransplant exendin-4 treatment in the recipient with a marginal number of fresh islets expands graft  $\beta$ -cell mass and improves transplantation outcome.

## MATERIALS AND METHODS

### *Animals*

Per previous studies (16,17), male inbred C57BL/6 mice (National Laboratory Animal Center, Taipei, Taiwan), aged 8–12 weeks, were used as transplantation donors and recipients. The recipients were made diabetic by a single IP injection of streptozotocin (STZ, Sigma Immunochemicals, St. Louis, MO, USA; 200 mg/kg body weight, freshly dissolved in citrate buffer, pH 4.5). Before transplantation, diabetes was confirmed by the presence of hyperglycemia, weight loss, and polyuria. Only those mice with blood glucose above 350 mg/dl at 2 weeks after STZ injection underwent transplant. Blood glucose values were determined on blood obtained from the tail incision, with measurements performed with a portable glucose analyzer (One Touch II, Lifescan Inc., Milpitas, CA, USA). The animal experiments were approved by the local animal ethics committee.

### *Islet Isolation*

Under anesthesia with sodium amobarbital, pancreases were distended with 2.5 ml of RPMI-1640 medium (GIBCO BRL, Grand Island, NY, USA) containing 1.5 mg/ml of collagenase (collagenase from *Clostridium histolyticum*, type XI, Sigma Immunochemicals), excised, and incubated in a water bath at 37°C. The islets were separated by a density gradient (Histopaque-1077; Sigma Immunochemicals), and purified islets were then handpicked under a dissecting microscope (16,17). Islets >75 and <250  $\mu$ m in diameter were collected and carefully counted into groups of 150 islets.

### *Islet Transplantation*

One hundred and fifty C57BL/6 mouse islets were syngeneically transplanted under left kidney capsule of the inbred streptozotocin-diabetic mice on the same day as the isolation. Blood glucose and body weight were measured periodically after transplantation and normoglycemia was defined as nonfasting blood glucose levels <200 mg/dl (16,17).

### *Exendin-4 Treatment*

After islet transplantation, 16 recipients were treated with exendin-4 (Sigma Immunochemicals), 3  $\mu$ g/kg bid SC, for 6 weeks. Seventeen recipients that had not received exendin-4 served as controls (11).

### *Intraperitoneal Glucose Tolerance Test (IPGTT)*

After an overnight fast, a 5% glucose solution (1.5 g/kg) was injected intraperitoneally, and blood glucose was measured at 0, 30, 60, 90, and 120 min by tail snipping. The IPGTT was performed at 2, 4, and 6 weeks after transplantation (15,17).

### *Removal of the Islet Graft*

Six weeks after transplantation, animals intended for graft removal were anesthetized with amobarbital. An abdominal incision was made and the kidney was exposed. Under dissecting microscope, the kidney capsule surrounding the graft was excised and removed with the adherent graft (15). The weight of each graft was determined on a Mettler balance type AE200 (Mettler Instruments Corp., NJ, USA).

### *Immunohistochemistry and $\beta$ -Cell Mass of the Islet Graft*

The removed grafts were fixed in formalin solution and processed for paraffin embedding and sectioning. Sections of grafts were stained for the endocrine  $\beta$ -cells with immunoperoxidase by a guinea pig anti-swine insulin antibody (Dako Co., Ltd, Denmark). Graft  $\beta$ -cell mass was measured by point counting morphometry on immunoperoxidase-stained sections. Each section was covered systematically using a 48-point grid to obtain the number of intercepts over  $\beta$ -cells, endocrine non- $\beta$ -cells, and other tissue. The  $\beta$ -cell relative volume was calculated by dividing the intercepts over  $\beta$ -cells by intercepts over total tissue;  $\beta$ -cell mass was then estimated by multiplying  $\beta$ -cell relative volume by graft weight (15–17).

### *Insulin Content of the Pancreas*

At 6 weeks after transplantation, the pancreases of normoglycemic recipients were removed, minced, and homogenized in acid ethanol. After homogenization, the samples were extracted overnight at 4°C. On the follow-

ing day, they were centrifuged at 2400 rpm for 30 min, and the supernatant was stored at  $-20^{\circ}\text{C}$ . The pellet was rehomogenized in acid ethanol and insulin extracted overnight. After centrifugation, this second supernatant was added to the first extraction sample and kept in a  $-20^{\circ}\text{C}$  freezer until assay. Insulin was measured by radioimmunoassay (rat insulin RIA, Linco Research, St. Charles, MO, USA). Because the mouse with pancreatic insulin of 83.44 ng was still diabetic in this series,  $\beta$ -cell regeneration in the endogenous pancreas was considered insignificant if the mouse pancreas had value equal or below this cutoff. The mice with the evidence of endogenous  $\beta$ -cell regeneration were excluded from the study.

#### Statistical Analysis

Results were expressed as mean  $\pm$  SD. Unpaired Student's *t*-test, chi square test, and Kaplan-Meier curve were employed to compare two groups. A value of  $p < 0.05$  was considered significant.

## RESULTS

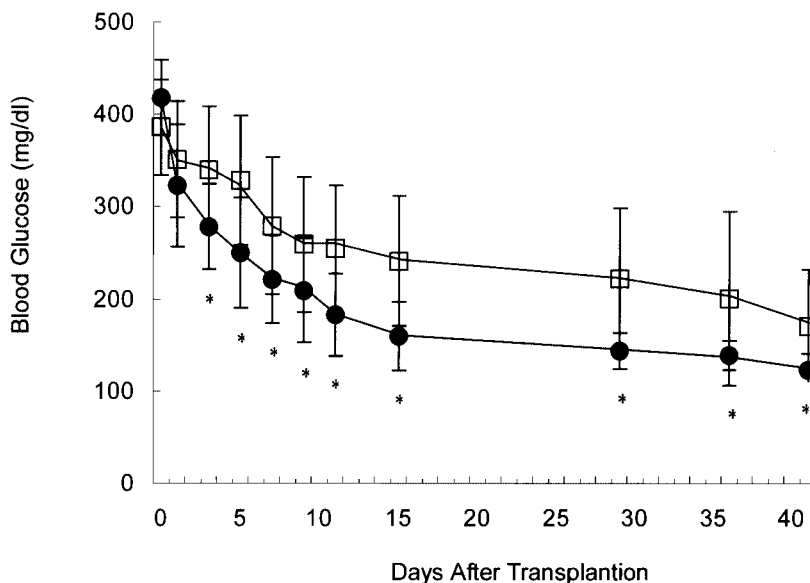
The possibility of  $\beta$ -cell regeneration in the endogenous pancreas was evaluated by measuring pancreatic insulin content in recipients achieving normoglycemia. One recipient in each group had pancreatic insulin content greater than 83.44 ng and were excluded from the study. Finally, there were 15 and 16 animals in exendin-4-treated group and controls, respectively.

#### Metabolic Evolution of Recipients After Islet Transplantation

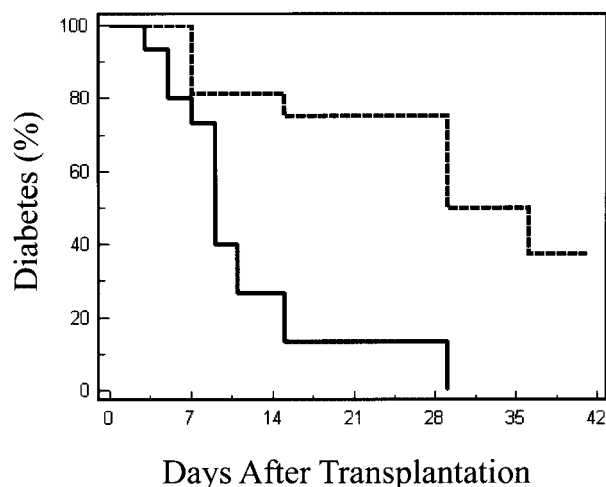
Blood glucose levels in both groups decreased progressively after transplantation. However, the exendin-4-treated group had lower blood glucose than controls (Fig. 1). By 6 weeks, euglycemia was achieved in 15/15 (100%) of mice treated with exendin-4 versus 10/16 (62.5%) of controls ( $p = 0.018$ ) (Fig. 2). Normoglycemia was observed at  $12 \pm 8$  days in the exendin-4-treated group and at  $29 \pm 13$  days in controls ( $p = 0.000$ ). Blood glucose at 6 weeks was  $123 \pm 18$  and  $170 \pm 62$  mg/dl in the exendin-4-treated group and controls, respectively ( $p = 0.008$ ). Additionally, the exendin-4-treated group had better glucose tolerance than the control group at 2 and 4 weeks ( $p < 0.02$ ) (Fig. 3). However, in both groups, body weight increased over time, and the change in body weight did not significantly differ between the two groups throughout the study period. At 6 weeks, body weight was  $24.8 \pm 1.0$  and  $24.4 \pm 2.3$  g ( $p = 0.562$ ), and weight gain was  $3.4 \pm 1.7$  and  $2.6 \pm 1.9$  g ( $p = 0.198$ ) in the exendin-4-treated group and controls, respectively.

#### Morphology and $\beta$ -Cell Mass of the Graft

At 6 weeks after transplantation, grafts in the exendin-4-treated group were more prominent than those of controls. Graft immunohistochemistry revealed a larger number of insulin-stained cells in the exendin-4-treated

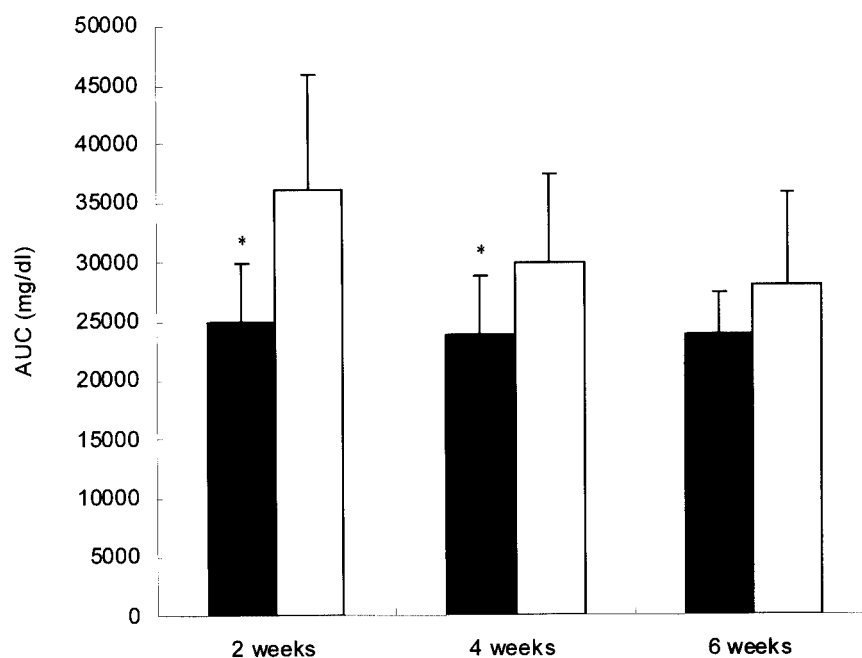


**Figure 1.** Evolution of blood glucose after islet transplantation in the exendin-4-treated (circles,  $n = 15$ ) and control (squares,  $n = 16$ ) groups. Data are expressed as mean  $\pm$  SD. \* $p < 0.05$  vs. control.



**Figure 2.** Kaplan-Meier curve of the percentage of diabetes over time in the exendin-4-treated (solid line,  $n = 15$ ) and control (dotted line,  $n = 16$ ) groups.  $p < 0.0002$  between the two groups.

group than in controls (Fig. 4). The graft  $\beta$ -cell mass was  $0.30 \pm 0.11$  mg (0.19–0.41 mg,  $n = 4$ ) in exendin-4-treated group and  $0.13 \pm 0.03$  mg (0.11–0.19 mg,  $n = 5$ ) in controls ( $p = 0.012$ ). Exendin-4-treated group had 2.3-fold more  $\beta$ -cell mass of the graft than controls.

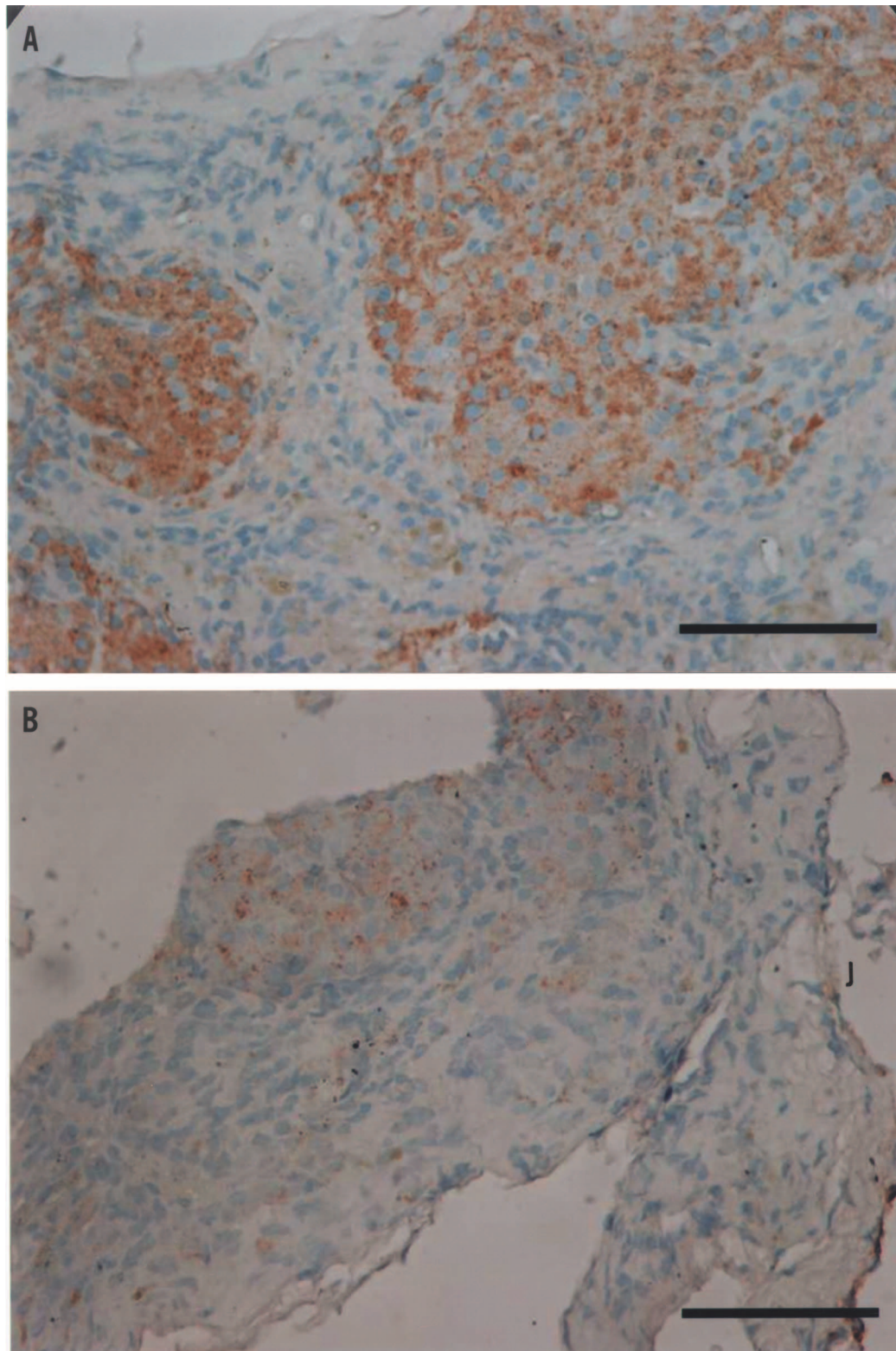


**Figure 3.** Intraperitoneal glucose tolerance at 2, 4, and 6 weeks after islet transplantation in the exendin-4-treated (filled columns,  $n = 15$ ) and control (open columns,  $n = 16$ ) groups. AUC: area under the curve. Data are expressed as mean  $\pm$  SD. \* $p < 0.02$  versus control.

## DISCUSSION

This study employed a syngeneic transplantation model to test whether exendin-4 treatment improves the outcome of transplantation with marginal number of islets. Previously, we demonstrated that, after syngeneic transplantation with 150 freshly isolated C57BL/6 mouse islets, 18% and 73% achieved normoglycemia at 4 and 12 weeks, respectively (16). In the present study, after transplantation with this suboptimal number of islets, the blood glucose levels in diabetic recipients decreased progressively. However, the exendin-4-treated group had not only lower blood glucose than controls but also better glucose tolerance at 2 and 4 weeks. By 6 weeks, more (100%) mice treated with exendin-4 than controls (62.5%) achieved euglycemia ( $p = 0.018$ ). Moreover, the time to obtain normoglycemia was much shorter in the former than in the latter ( $12 \pm 8$  vs.  $29 \pm 13$  days,  $p = 0.000$ ). These observations indicate exendin-4 treatment in diabetic recipients transplanted with a suboptimal number of islets improves glycemia, increases the cure rate of diabetes, and reduces the time needed to cure diabetes.

Sharma et al. demonstrated exendin-4 treatment improved recipients' metabolic control by transplanting 30 rat islets precultured with exendin-4 to athymic mice and then further treated recipients with exendin-4 after transplantation (32). In that study, it is difficult to dis-



**Figure 4.** Immunohistochemistry with insulin staining of the exendin-4-treated (A) and control (B) grafts at 6 weeks after islet transplantation. Scale bar: 100 μm.

sect the beneficial effects of the exendin-4 treatment *in vitro* and *in vivo*. In contrast, our experiments using freshly isolated mouse islets clearly demonstrate that posttransplant exendin-4 treatment can improve islet recipients' glucose metabolism. However, our results are contradictory to those of King et al., who showed posttransplant exendin-4 treatment did not improve glucose homeostasis in the recipients initially transplanted with a large number (i.e., 500) of freshly isolated mouse islets (20). Because we found the exendin-4-treated group had lower blood glucose than controls soon after transplantation, the duration of exendin-4 treatment (2 vs. 6 weeks) cannot explain these differences. Strict metabolic control of diabetic animals, either by insulin treatment or by transplantation of additional islets, has been shown to enhance the growth and function of the islet graft (1,3,4,14,15,18,25,26). Thus, it is possible that the benefit of exendin-4 treatment was masked by the effects of normoglycemic environment achieved by transplantation of a large number of islets. In contrast, our recipients with a marginal number of islets remained hyperglycemic for a period after transplantation, so that the exendin-4 could exhibit its advantages.

Exendin-4 improves glycemic control in type 2 diabetic patients by stimulating glucose-dependent insulin secretion and biosynthesis as well as by suppressing glucagon secretion, gastric emptying, and appetite (8,9). In the present study, body weight in recipients with and without exendin-4 treatment gradually increased after transplant and the two groups did not significantly differ in weight change throughout the study period. These findings are consistent with those reported by King et al. (20), presumably because improved glucose homeostasis alone can cause weight gain despite the suppressive effects of exendin-4 on appetite and gastric emptying (8,9). From the observation on recipients' weight, we speculate that increased insulin secretion and biosynthesis as well as suppression of glucagons secretion, instead of decreased food intake and delayed gastric emptying, play a major role in lowering blood glucose in exendin-4-treated recipients.

In the present study, the possibility of  $\beta$ -cell regeneration in the endogenous pancreas was excluded. Therefore, the metabolic improvements in our recipients are most likely contributed by transplanted  $\beta$ -cells. At 6 weeks after transplantation, the  $\beta$ -cell mass in the exendin-4-treated group was 2.3-fold that of controls. It is comparable with previous studies that showed exendin-4 treatment in rodent leads to a 1.4–6.2-fold increase in  $\beta$ -cell mass (2). The ability of exendin-4 to increase  $\beta$ -cell mass by stimulating  $\beta$ -cell replication and neogenesis (2,21,23,27,35,36) as well as preventing  $\beta$ -cell apoptosis (2,5,22,33) has been demonstrated in studies using islet and  $\beta$ -cell primary cultures or cell lines as well as in experiments on normal and diabetic rodents. Because we

transplanted isolated islets instead of pancreatic clusters, the increased  $\beta$ -cell neogenesis from precursor or stem cells is excluded. It has previously been shown that the first few days of islet transplantation are characterized by dynamic changes, with substantial islet cell dysfunction and death followed by tissue remodeling and then stable engraftment (3,6). Thus, exendin-4 may inhibit  $\beta$ -cell apoptosis early after islet transplantation and stimulate  $\beta$ -cell replication at a later stage. It is also possible that all of the beneficial effects in the exendin-4 group were a consequence of better glycemic control achieved by exendin-4 treatment. Further studies are needed to learn the mechanisms of exendin-4 on islet grafts.

In summary, our findings indicate posttransplant exendin-4 treatment can expand graft  $\beta$ -cell mass and improve transplantation outcome in diabetic mice with a marginal number of fresh islets. Recent studies in type 1 diabetes demonstrated that transplanted human islets retain the ability to response to GLP-1 (10) and exenatide stimulates insulin secretion in islet recipients (12). Thus, exendin-4 has clinical potential to increase the success rate of islet transplantation, minimize the number of islets required to reverse diabetes and, hopefully, prolong insulin independence after islet transplantation.

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