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Therapeutic Effects of a Non-Beta Cell Bioartificial Pancreas in Diabetic Mice

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Abstract

Background—Cell-based insulin therapies can potentially improve glycemic regulation in insulin dependent diabetes patients. Enteroendocrine cells engineered to secrete recombinant insulin have exhibited glycemic efficacy, but have been primarily studied as uncontrollable growth systems in immune incompetent mice. Furthermore, reports suggest that suboptimal insulin secretion remains a barrier to expanded application.

Methods—Genetic and tissue engineering strategies were applied to improve recombinant insulin secretion from intestinal L-cells on both a per-cell and per-graft basis. Transduction of insulin-expressing GLUTag L-cells with lentivirus carrying an additional human insulin gene enhanced secretion 2-fold. We infected cells with lentivirus expressing a luciferase reporter gene to track cell survival *in vivo*. To provide a growth-controlled and immune protective environment without affecting secretory capacity, cells were microencapsulated in barium alginate. Approximately 9×10^7 microencapsulated cells were injected intraperitoneally in immune competent streptozotocin-induced diabetic mice for therapeutic efficacy evaluation.

Results—Graft insulin secretion was increased to 16–24 mU insulin/day. Transient normoglycemia was achieved in treated mice two days after transplantation, and endogenous insulin was sufficient to sustain body weights of treated mice receiving minimal supplementation.

Conclusions—Glycemic efficacy of a bioartificial pancreas based on insulin-secreting enteroendocrine cells is insufficient as a standalone therapy, despite enhancement of graft insulin secretion capacity. Supplemental strategies to alleviate secretion limitations should be pursued.

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Keywords

bioartificial pancreas; β -cell replacement therapy; enteroendocrine cells; alginate microcapsules

Introduction

Of the approximate 25.8 million people in the United States with diabetes mellitus, 6.7 million have insulin dependent diabetes (IDD) (1). To prevent long-term complications in patients with IDD, blood glucose levels must be tightly regulated with multiple daily insulin injections or continuous subcutaneous insulin infusion (CSII) (2). Even with diligent monitoring, many patients fail to achieve recommended blood sugar control. There is a clear need for treatments that improve glycemic regulation, reduce morbidity, and improve quality of life.

Pancreatic islet transplantation has addressed this need, but widespread use is hindered by islet scarcity and chronic immune rejection (3). To overcome these hurdles, researchers have explored the use of a replacement therapy with autologous non- β cells engineered for meal or glucose-responsive insulin secretion. Three enteroendocrine cell types, gastric G-cells (4), intestinal L-cells, and intestinal K-cells (5), inherently express the necessary prohormone convertases for proinsulin to insulin processing and possess a secretory mechanism similar to β -cells, making them promising β -cell surrogate candidates.

Initial proof-of-concept studies showed that mice transgenic for human insulin-secreting K-cells were resistant to streptozotocin (STZ)-induced diabetes (4). Similarly, insulin-secreting G-cells significantly reduced mouse blood glucose levels (6). The engineering of available immortalized enteroendocrine cell lines for insulin secretion has permitted preclinical evaluation without resorting to transgenics. For example, insulin-secreting STC-1 K-cell clones transplanted into diabetic mice restored normoglycemia (7, 8). However, treated mice became hypoglycemic as unrestricted cell proliferation increased insulin production beyond therapeutic levels. Additionally, immune incompetent mice were used as the preclinical model to avoid transplant rejection.

Encapsulation can be utilized to control cell growth and provide immune protection in immune competent mice. However, Unniappan et al. found that microencapsulated insulin-producing K-cells were unable to control blood sugars until a drug-inducible element was incorporated into the insulin transgene (9). Alternatively, Bara et al. seeded human insulin-secreting L-cells in a macrocapsule device for transplantation (10–12), but blood glucose levels were unaffected. Insufficient insulin secretion and limitations imposed by macrocapsules were two likely barriers that prevented these systems from serving as standalone diabetes treatments.

Here we report the therapeutic effects of an enhanced insulin-secreting L-cell line, based on the previously developed recombinant GLUTag-INS cell line characterized in (10), that was microencapsulated in alginate and transplanted intraperitoneally (i.p.) in STZ-diabetic mice. We discuss the implications of this study in the context of a standalone enteroendocrine cell

therapy and the potential benefit of a dual cell therapy for complete normoglycemic restoration.

Results

Pancreatic substitute fabrication

GLUTag-EINS cells (EINS) exhibited 1.7 and 2-fold enhancements in stimulated and basal insulin secretion rates (ISR) per cell relative to GLUTag-INS. GLUTag-EINS-Fluc cells (Fluc) stably expressed bioluminescence and retained EINS insulin secretory capacity (Figure 1A).

Microencapsulation had no effect on insulin secretion per cell and no statistical difference existed between EINS and Fluc secretion one day post-encapsulation (Figure 1B). However, 14 days post-encapsulation, Fluc microcapsules secreted less per volume (Figure 1C) while the stimulation index remained the same (Figure 1D). Consistent with lower insulin output per microcapsule, Fluc exhibited 52% less metabolic activity, as assessed by alamarBlue™, than EINS on day 14 (data not shown), possibly due to slower Fluc cell growth. These data instigated the fabrication of a mixed microcapsule system consisting of mostly EINS to maximize insulin output and enough Fluc to retain BLI monitoring capabilities.

Estimates of transplant volumes to normalize glycemia were based on reported insulin secretion from microencapsulated β TC-tet cells that restored normoglycemia in STZ-induced diabetic mice (13). EINS data from Figure 1C indicated that 7.5 mL microcapsules would be sufficient to control glycemia, but due to space restrictions, 3 mL (corresponding to 9×10^7 EINS cells) was the maximum volume attempted for transplant in mice. Consequently, lowered blood glucose levels in diabetic mice without complete normoglycemic restoration was expected.

The mixed microcapsule ratio was determined based on a previous initial BLI characterization study, where 1 mL of microencapsulated Fluc cells produced abundant BLI signal for cell monitoring *in vivo* (data not shown). Since the total transplant volume was fixed at 3 mL, a 1:2 ratio of Fluc:EINS microcapsules was chosen.

Therapeutic efficacy evaluation

Diabetic mice were injected i.p. at a 1:2 ratio of Fluc:EINS microcapsules. Strikingly, experimental mice became normoglycemic two days later, whereas control mice remained hyperglycemic (Figure 2A); neither group received exogenous insulin before day 2 (Figure 2B). Correction was short-lived however, and mice reverted to hyperglycemia by day 4. Control mice exhibited significantly higher blood glucose levels than experimental mice on one other day of the study (day 8). Overnight fasting revealed higher blood glucose levels in controls compared to experimental mice on day 17; exogenous insulin was withdrawn 3–4 days before fasting.

Figure 2B indicates the average daily exogenous insulin administration; overall, control mice received 7.8-fold more insulin ($p < 0.001$). No differences were detected in body weight trends between the two groups on any day of the study (Figure 2C). Figures 2D-E depict

dynamic BLI signals, indicating proliferation from day 3 to 8 and likely some cell death from day 8 to 17.

Explant analyses

On day 17, mice were euthanized, 0.5–1 mL blood was collected via cardiac puncture, and approximately 90% of the microcapsules were retrieved for analyses under the assumption that microcapsules remained at a 1:2, Fluc:EINS ratio. No difference in BLI was found between explanted ($2.6 \pm 0.3 \times 10^8$ photons/s) and *in vitro* control groups ($2.2 \pm 0.9 \times 10^8$ photons/s); live/dead images also indicated similar viability between groups (Figure 3A). Light microscopy images showed that microcapsule structural integrity was maintained *in vivo* and that the microcapsule periphery was free of host cell adhesion (Figure 3B), also corroborated by H&E histology (Figure 3C). Figure 3 images indicated relatively uniform distribution of cells within the microcapsules; explants appeared to have higher cell densities relative to *in vitro* controls. Since blood glucose levels near the end of the study indicated that mice did not endogenously produce sufficient insulin, pancreas collection and insulin staining were not performed.

Interestingly, explanted cells were 2.7 times less metabolically active with a 3-fold lower insulin secretion capacity compared to *in vitro* controls (Figure 4A-B). Therefore, when normalized to metabolic activity, there was no difference in ISR between groups (Figure 4C). Similarly, explant intracellular insulin content was 2-fold lower than *in vitro* controls (Figure 4D), but no different when normalized to metabolic activity (Figure 4E). Serum human insulin concentrations were high in experimental mice, ranging from 12–62 $\mu\text{U/mL}$, and close to zero in controls (Figure 4F).

Discussion

Enhancement and microencapsulation of the previously engineered GLUTag-INS cells produced a graft that secreted roughly 100-fold more insulin per unit graft volume than the macrocapsule formerly developed (11). Even with dramatic secretion augmentation, microcapsules only restored normoglycemia for two days post-transplantation in diabetic mice, after which hyperglycemia was not completely corrected (14). Treated mice did, however, maintain body weight and avoid extreme hyperglycemia with approximately 8-fold less exogenous insulin than controls. Fasting blood glucose levels and serum human insulin concentrations on day 17 revealed a still-functioning graft *in vivo*; BLI demonstrated temporal survival of the grafts.

Reduced food consumption during post-surgical recovery likely contributed to the normoglycemia of treated mice on day 2 with insulin secretion from grafts being temporarily sufficient to control blood glucose levels. A less likely possibility is that the graft was initially effective, but secretory function subsequently declined to a non-therapeutic level, resulting in recurrence of hyperglycemia. BLI data, however, contradicted this rationale by indicating an approximate 2-fold increase in viable cells from day 3 to 7. It therefore seemed unlikely that cells, during such a proliferative time, would experience secretion impairment.

Fasting blood glucose data collected from experimental and control mice were similar to those reported in the literature for normal and STZ-diabetic mice, respectively (15). Overnight fasting elucidated that ad libitum feeding contributed significantly to hyperglycemia; a difference between groups is therefore more likely with controlled food consumption. From these findings, it was reasonably inferred that grafts did not secrete sufficient insulin to overcome extreme hyperglycemia in freely eating diabetic mice.

The average human insulin serum concentration in treated mice was 9-fold higher than that reported in diabetic mice treated with GLUTag-INS macrocapsule constructs, and similar to murine insulin concentrations in healthy mice (12). Blood insulin concentration profiles were generated to determine whether serum insulin measurements were reasonable (Figure 5). Profiles were produced based on ISR data from explanted grafts (969 $\mu\text{U}/\text{h}$), insulin clearance rates (16), and assuming all insulin secreted from grafts appeared in the blood. Clearance rates of 13 and 63 mL/h were estimated by proportionally scaling down, based on body weight, from the physiological range of insulin clearance in a 70 kg human (700–3350 mL/min) (16) to a 22 g mouse. Since scaling down to rats is approached in this way (17), using the same method to estimate mouse clearance rates was assumed acceptable. The steady state blood insulin concentration ranged between 15 and 75 $\mu\text{U}/\text{mL}$; the average measured serum insulin concentration from mice on day 17 (50 $\mu\text{U}/\text{mL}$) was indeed within this range.

The effects of mouse insulin antibodies are unknown, but interference from human insulin autoantibodies produce falsely high measurements in human insulin RIAs (18). It is also documented that human insulin may be immunogenic in BALB/c mice (19), but in the present study, control mice receiving significant amounts of exogenous human insulin had negligible insulin serum concentrations measured by RIA. However, since the response in transplant groups may not be equivalent to controls, interference cannot be completely ruled out. Nevertheless, serum human insulin was within the expected concentration range and insulin activity in treated mice was evident from their sustained body weight and blood glucose trends. Moreover, treated mice received almost 8-fold less insulin supplementation. Importantly, even though human insulin-secreting grafts are commonly used, diabetic rodent models have shown resistance to exogenous porcine and human insulin, requiring 20–40 times more porcine insulin than the prescribed human dose (20). This challenge, in conjunction with overeating, may explain why normoglycemia was not achieved despite high serum human insulin levels.

Interestingly, explant tests revealed that *in vivo* conditions impaired both metabolism and secretory function without instigating significant cell death. Schneider et al. reported similar findings after transplanting microencapsulated islets in immune competent diabetic mice (21). After 10 weeks, viability was estimated at >85%, but insulin secretion capacity was significantly reduced. Skiles et al. saw a similar effect *in vitro* after culturing encapsulated MIN6 aggregates under hypoxia for weeks (22, 23). Although these findings relate to β -cells, L-cells also rely on glucose metabolism for stimulated secretion (24) which is disrupted under hypoxic conditions (25). Hyperglycemic stress as a potential cause for graft dysfunction in this study seemed unlikely since average blood glucose levels ($21 \pm 5.6 \text{ mM}$) were comparable to *in vitro* culture conditions (22 mM glucose) which had no effect on

function. Although smaller 1 mL volumes of Fluc-containing microcapsules have previously shown no evidence of function loss in normal mice (data not shown), the contributing factor was probably not the absence of hyperglycemia but the better oxygenated peritoneal cavity (13) as a result of transplanting 67% fewer cells than in the present study. Additional studies will need to be performed to confirm *in vivo* hypoxic effects on transplants, possibly by using the injectable hypoxia marker, pimonidazole. Nevertheless, simply transplanting higher cell numbers is not a plausible option to achieving therapeutic efficacy.

In this study, two aspects of pancreatic construct fabrication contributed to improved secretion: 1) Additional insulin gene introduction via lentivirus allowing for 1.7–2 times more insulin secretion and 2) microencapsulation for retaining monolayer secretion and allowing for the transplant of greater cell numbers. However, results imply that further progress is needed to establish a preclinically relevant enteroendocrine cell-based pancreatic construct. Although per-cell insulin secretion from engineered L-cells is estimated at less than 5% of that from mouse β -cells (26), we calculate that therapeutic efficacy can be achieved with a realistic 1 mL transplant volume if a 6-fold increase in insulin secretion is obtained.

An alternative method is to complement the graft from this study with a hepatic cell therapy. Hepatic cells genetically engineered for glucose-responsive insulin secretion (27, 28) can serve as the basal release portion of the biphasic insulin response and enteroendocrine cells can supply the quick-phase burst via their meal-responsive secretory granule system (29). This dual cell system may therefore accomplish both the level and kinetics of insulin release needed for improved glycemic regulation.

Materials and methods

Cell culture

GLUTag-INS (10), EINS, and Fluc cells were cultured as in (29) using Dulbecco's modified Eagle's medium (DMEM) with 25 mM glucose, without L-glutamine (Corning cellgro, Manassas, VA), and supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cultures were propagated in a humidified incubator at 37°C/5% CO₂. The EINS cell line was generated by transduction of GLUTag-INS with the wild-type human insulin lentivirus (WT-INS; Emory University; Dr. John Shires) and the Fluc cell line was generated by transduction of EINS with the firefly luciferase lentivirus (Capital Biosciences, Rockville, MD). Two days after seeding in 12-well plates at 1×10^5 cells/cm², lentivirus was added at a multiplicity of infection of 30 to fresh medium containing 8 μ g/mL of polybrene (Sigma). After 24 hours, the medium in wells was changed to fresh and cells were passaged, expanded, and conventionally frozen.

Pancreatic substitute fabrication

ISR tests were performed on EINS and Fluc cells passaged 3–7 times post-lentiviral transduction. Monolayers were seeded in 12-well plates and subjected to basal conditions (1 mL DMEM containing 5mM glucose) for two hours followed by a two hour step-up period under stimulating conditions (1 mL basal medium with 2% meat hydrolysate; Sigma).

Insulin measurements were made using an insulin RIA (Millipore, Billerica, MA) ($1 \mu\text{U/mL} = 6 \text{ pM}$). Cells were microencapsulated in 3.3% LVM alginate (LVM; Novamatrix, Drammen, Norway) and cross-linked in 30 mM BaCl_2 at an encapsulation density of 3×10^7 cells/mL alginate. Encapsulation was performed using an electrostatic droplet generator (Nisco Engineering, Zurich, Switzerland) as in (13) to produce microcapsules with diameters of approximately 500 μm , which previous studies showed are small enough to allow proper oxygenation to all cells in the capsule (30) and did not compromise insulin secretion dynamics shortly post-encapsulation (31, 32).

One and fourteen days post-encapsulation, ISR tests were performed on 0.1 mL volumes of microcapsules in 100 μm strainers placed in 12-well plates containing 5 mL of basal or stimulating media. Viable cell counts were only possible one day post-encapsulation, before cell aggregation within microcapsules prevented reliable hemocytometer cell counting. For this reason, metabolic activity measurement via alamarBlue™ (Life Technologies, Grand Island, NY) was used to track viable microencapsulated cells over time. After an ISR test, microcapsules were either solubilized for trypan blue (Sigma) viable cell counting or incubated in alamarBlue™ for one hour as in (33). Microcapsule solubilization was achieved in ethylenediaminetetraacetic acid disodium salt dihydrate solution (ED2SS; 0.2 M; pH 9) for three minutes.

***In vivo* efficacy evaluation**

All procedures were approved by the Georgia Institute of Technology's Animal Care and Use Committee. Six week old male BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Six days before transplantation, diabetes was induced by i.p. injection of 180 mg/kg streptozotocin (STZ; Sigma) solubilized in sodium citrate solution (22.5 g/L; pH 4.5). Lantus® insulin was injected subcutaneously based on a predetermined criteria.

All microcapsules were fabricated one day before transplantation. On day 0, aliquots of microencapsulated EINS (2 mL) and Fluc (1 mL) cells were mixed in five centrifuge tubes (3 mL each). Four tubes were prepared with 3 mL acellular microcapsules in each. Microcapsules were then washed eight times in 50 mL unsupplemented high glucose DMEM and loaded into 3 mL syringes. Five diabetic mice received the EINS/Fluc microcapsules (experimental group; $n=5$) and four received the acellular microcapsules (control group; $n=4$). Microcapsules were injected i.p. through an 18 gauge needle in mice under isoflurane anesthesia. Approximately 1 mL of the EINS/Fluc microcapsule mixture was cultured *in vitro* as a control, run parallel to *in vivo* groups.

Random, daily glucose concentrations were measured in blood samples collected from the tip of the mouse tail using a TRUEtrack glucose monitor (Nipro Diagnostics, Fort Lauderdale, FL); body weight was also measured daily. Mice were fed ad libitum until day 16 when mice were fasted overnight (16–18 hours) to examine effects of eating.

Exogenous insulin administration criteria

Exogenous insulin (1–2 U Lantus® insulin every two days as long as mice were diabetic (higher than 350 mg/dl)) was administered to maintain body weight and avoid extreme hyperglycemia (higher than 450 mg/dl). To avoid hypoglycemia (lower than 50 mg/dl) and adjust for insulin produced by the grafts, transplanted animals only received 1 U Lantus® for persistently extreme hyperglycemia.

Bioluminescence imaging

BLI was acquired from experimental mice on days 3, 8, 13, and 17 for minimally-invasive cell survival tracking *in vivo*. BLI was performed using the In Vivo Imaging System (IVIS) Lumina (Perkin Elmer, Grayson, GA). Mice were placed under isoflurane anesthesia (1.2%) and injected i.p. with 200 mg/kg D-luciferin (RPI Corp, Mount Prospect, IL) five minutes before an image acquisition of less than 20 seconds. Bioluminescence data are reported in photons/s.

Explant analyses

Mice were euthanized on day 17 and serum was collected by cardiac puncture, incubated in CAPIJECT® blood collection tubes with no additives at room temperature for 20 minutes, and centrifuged for 10 minutes at 4° C and 1,600 xg for supernatant collection and insulin measurement. Grafts were retrieved by making incisions through the skin and muscle of the abdomen and collecting microcapsules with a pipette following peritoneal washes. Explant assessments included ISR and intracellular insulin measurement, BLI, metabolic activity measurement, live/dead analysis, light microscopy imaging, and hematoxylin/eosin (H&E) histological analysis. Live/dead analysis was performed by following the manufacturer's protocol (LIVE/DEAD® Viability/Cytotoxicity Kit; Life Technologies). Histology was performed as in (34) with resin embedding.

Statistical analysis

All data were analyzed using Minitab software (Minitab, Inc., State College, PA) and reported as mean $\pm$ standard error; each mean was the average of data from three or more independent experiments or mice. Normality of data was confirmed using the Anderson-Darling Normality Test, with normality defined as $p > 0.10$. Significance was determined using a one-way analysis of variance (ANOVA) with the general linear model, with significance defined as $p < 0.05$.

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Abbreviations

BLI	Bioluminescence Imaging
IDD	Insulin Dependent Diabetes
i.p.	Intraperitoneal
ISR	Insulin Secretion Rate
RFU	Relative Fluorescence Units
STZ	Streptozotocin

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Figure 1.

In vitro pancreatic substitute fabrication and characterization. A) Basal and stimulated ISR from GLUTag-INS, GLUTag-EINS (EINS), and GLUTag-EINS-Fluc (Fluc) cell monolayers. B) ISR normalized to viable cell number from microencapsulated EINS and Fluc cells one day post-encapsulation. C) ISR per mL volume of microencapsulated EINS and Fluc cells 1 and 14 days post-encapsulation (d1 & d14). D) Stimulation indices (stimulated/basal ISR) of microencapsulated EINS and Fluc cells 1 and 14 days post-encapsulation. In Figure A, asterisks indicate a statistical difference from GLUTag-INS under each condition ($*p<0.05$). For each group, secretion under stimulated conditions was statistically higher than basal ($p<0.05$). In Figures B-C, comparisons were made only between groups under each condition and asterisks indicate statistical differences from GLUTag-EINS ($*p<0.05$). In Figure D, all data were compared and no statistical differences found.

Figure 2.

In vivo therapeutic efficacy evaluation. A) Average daily blood glucose measurements for control mice receiving acellular microcapsules and experimental mice receiving EINS:Fluc microcapsules at a 1:2 ratio. B) Average exogenous Lantus[®] insulin administered per day for control and experimental mice. C) Average daily body weight normalized to day -8, for control and experimental groups. D) *In vivo* BLI signals obtained from experimental mice. E) Representative bioluminescence images of experimental mice over time post-transplantation (d3, d8, d13, d17). In Figures A-C, * indicates a significant difference between the control and experimental groups on that day ($p<0.05$). In Figure D, asterisks indicate statistical difference from Day 3 ($*p<0.05$).

Figure 3.

Qualitative assessment of explanted pancreatic substitutes from treated mice. Representative images of explanted microencapsulated EINS/Fluc cells compared to a parallel *in vitro* control group on day 17 using A) Confocal LIVE/DEAD, B) light microscopy, and C) H&E histological analyses.

Figure 4.

Quantitative explant analyses on day 17. A) Metabolic activity, measured by alamarBlue™ in relative fluorescence units (RFU), per mL explanted microcapsules compared to *in vitro* controls, B) ISR per mL explanted microcapsules versus *in vitro* controls, and C) ISR per mL microcapsules normalized to metabolic activity. D) Intracellular insulin content per mL explanted versus *in vitro* microcapsules and E) intracellular insulin normalized to metabolic activity. F) Human insulin concentrations in serum collected from control and experimental mice. In Figure B, stimulated insulin secretion rates were statistically higher than basal for each group ($p<0.05$). In Figures A-E, * indicates statistical difference from the corresponding *in vitro* group (* $p<0.05$). In Figure F, ** indicates statistical difference from the Control group (** $p<0.05$).

Figure 5.

Blood insulin concentration profiles based on insulin secretion data from explanted grafts, estimated insulin clearance rates in mice, and the assumption that all insulin secreted from grafts appeared in the blood stream. The following equation was generated to obtain profiles for both fast ($c=63$ mL/h) and slow ($c=13$ mL/h) clearance rates:

$Ci = \frac{isr}{c} + (Ci(0) - \frac{isr}{c}) \cdot e^{-c \cdot t}$, where Ci =blood insulin concentration, isr =explanted graft insulin secretion rate (969 μU/h), c =clearance rate (mL/h), and t =time (h). An arbitrary initial blood insulin concentration of 150 μU/mL was chosen and the total blood volume of a mouse was estimated to be 1 mL (the National Centre for the Replacement Refinement & Reduction of Animals in Research estimates that mice have around 58 mL of blood per kg of body weight).