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ORIGINAL ARTICLES

A Cell-Based Approach for Diabetes Treatment Using Engineered Non-β Cells

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Abstract

Background:

Implantation of insulin-secreting cells has the potential to provide tight glycemic regulation in diabetic subjects. Implantation of cadaveric human islets in immunosuppressed human patients is currently applied at a very small scale. To overcome the limitations of tissue availability and recipient immunosuppression, encapsulation of nonautologous cells and use of potentially autologous nonislet cells, the latter engineered for insulin secretion, are being pursued. This article reports on recent findings with the implantation of tissue constructs containing enteroendocrine cells stably expressing recombinant insulin in diabetic mice. The concept of a dual recombinant hepatic and enteroendocrine cell system, which may better approximate the secretory response of islets, is discussed.

Methods:

Mouse GLUTag-INS cells engineered to secrete human insulin were developed and incorporated in tissue constructs as reported previously. Constructs were implanted intraperitoneally in diabetic mice, and blood glucose levels, animal weights, and plasma insulin levels were measured at various time points.

Results:

GLUTag-INS-containing tissue constructs secreted insulin preimplantation and postexplantation, and human insulin was detected in the plasma of diabetic mice. However, normoglycemia was not restored.

Conclusions:

A variety of cell types and of encapsulation methods to enhance immune acceptance of insulin-secreting grafts are being pursued. Recombinant enteroendocrine cells show promise, but it is likely that they need to be combined with recombinant hepatic cells to achieve glycemic normalization.

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Abbreviations: (IDD) insulin-dependent diabetes, (GLP-1 and -2) glucagon-like peptide-1 and -2, (mRNA) messenger ribonucleic acid, (PC) prohormone convertase, (RIA) radioimmunoassay, (SIS) small intestinal submucosa, (STZ) streptozotocin

Keywords: β-cell surrogates, enteroendocrine cells, GLUTag, hepatocytes, insulin secretion dynamics, tissue engineering

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Introduction

The poor temporal match between glucose load and insulin action leads to a number of serious complications.[1] A study by the Diabetes Control and Complications Trial Group[2] suggested that while aggressive monitoring and insulin therapy could postpone the onset of secondary complications, it could not prevent them and increased the occurrence and severity of hypoglycemic episodes. Because cells can release insulin in response to physiologic cues, a cell-based therapy, which provides continuous regulation of blood glucose, has the potential to provide a much tighter control of blood glucose levels.

The application of allogeneic islet transplantation is restricted to a small subset of the diabetic population due to a limited supply of cadaveric donor pancreases, with two or more pancreases used to treat a single patient, and the need for patient immunosuppression. The islet transplantation protocol developed at the University of Edmonton[3,4] improved the survivability of allogeneic islet grafts dramatically, with 80% of patients being insulin independent 1 year post-transplantation. Just 5 years after transplantation, however, only 10% of patients remained insulin independent.[5] Immunosuppressive therapy, which is necessary to prevent rejection, is one of the biggest challenges with islet transplantation. Immunosuppressive drugs have diabetogenic properties and may account for some of the progressive loss of β cells in the graft.[5,6] Specific immunosuppressive drugs may have additional effects, such as nephrotoxicity.[5,6] Current work focuses on alleviating the two major limitations of allogenic islet transplantation: cell/tissue availability and immune suppression.

Genetically Engineered Non-β Cells

In addition to human islets, investigators are also exploring the use of animal, especially porcine, islets and of cell lines as a means to relax the cell availability limitation.[7] Due to the restrictions of allogeneic or xenogeneic transplantation, which include limited supply and immunogenicity, a potentially autologous cell source is especially attractive. Use of non-β cells from the patients themselves relaxes the requirements of availability and immune acceptance. It should be noted that expressing recombinant insulin in non-β cells is straightforward; however, achieving the correct kinetics is particularly challenging, as the regulatory system of insulin biosynthesis and secretion inherent to β cells is complex. While insulin is a suspected trigger for autoimmunity in type 1 diabetes because its encoding gene is only expressed in cells that are specifically targeted during autoimmune attack,[8] given the current understanding of autoimmunity in diabetes, expression of insulin alone in non-β cells is not expected to trigger the autoimmune response. Indeed, multiple islet molecules are the target of autoimmunity in human and in animal models of type 1 diabetes.[9–11] In transgenic nonobese diabetic mice that secreted mature insulin from pituitary cells, these cells were not targeted or destroyed by the immune system, unlike pancreatic β cells,[12] suggesting that the expression of insulin alone in non-β cells is not sufficient to induce autoimmune destruction, at least in this model for autoimmune diabetes.

Like β cells, liver cells (hepatocytes) express both the glucose transporter GLUT2 and the glucose-phosphorylating enzyme glucokinase, components of a glucose sensing system, making them likely candidates for regulated recombinant insulin expression. Although hepatocytes do not have the specific endoproteases necessary to process wild-type proinsulin, recombinant insulin that is cleaved by the ubiquitous endopeptidase furin[13] has enabled successful expression of active insulin in these cells.

Enteroendocrine cells secrete hormones in a tightly controlled manner that parallels the secretion of insulin by β cells, following oral glucose load.[14] L cells produce glucagon-like peptide-1 and -2 (GLP-1 and GLP-2) and secrete them in parallel in adult humans after oral feeding. GLP-1 and GLP-2 are released in a biphasic manner with a rapid initial increase occurring within 15 minutes, followed by a second increase after about 1 hour poststimulation.[15] Because of the unique connection between incretins and insulin, engineering enteroendocrine cells for regulated insulin secretion arises as an appealing approach for insulin-dependent diabetes (IDD) treatment in terms of the dynamic release of insulin, as well as the compatibility of incretins in glycemic normalization. Work by Cheung and colleagues in 2000 demonstrated that insulin produced and secreted by intestinal K cells in transgenic mice prevented these mice from becoming diabetic after injection with streptozotocin (STZ).[16] Similarly, transgenic mice that produced human insulin in gastric G cells displayed a meal-regulated increase in the level of recombinant insulin and a corresponding decrease in blood glucose levels.[17] These are important proof-of-concept studies, which showed

that enteroendocrine cell-produced insulin can provide regulation of blood glucose levels.

In our laboratories, we focus on cell-based therapies using potentially autologous liver and enteroendocrine cells. Use of liver cells, engineered genetically with furin-compatible insulin under transcriptional regulation, is advantageous as hepatocytes are numerous and can be targeted easily by viral vectors. These cells have inherently slow kinetics, however, as the absence of secretory granules does not provide for acute insulin secretion in response to induction, and the downregulation of insulin secretion upon lowering of the blood glucose level is also slow.[18,19] As such, intestinal endocrine cells, which do express the prohormone convertases (PC) PC1/3 and PC2 and secrete endogenous products that are compatible with prandial metabolism in a nutrient-responsive manner, may complement recombinant hepatic function.

Viral delivery to the liver of diabetic mice of an insulin transgene that is upregulated by glucose and downregulated by insulin achieved at least some normalization of the animals' glycemic state.[20–23] To improve the kinetics of downregulation of insulin secretion of transcriptionally regulated cells, the insulin messenger ribonucleic acid (mRNA) was destabilized, resulting in a faster decline of insulin secretion upon downregulation of transcription. Initial work was performed with recombinant human hepatoma cells[19] and subsequently with primary hepatocytes in culture.[18] Importantly, expressing destabilized insulin mRNA in the liver of STZ diabetic mice significantly reduced, if not eliminated, the hypoglycemic excursions observed following an intraperitoneal glucose bolus in animals expressing the stable mRNA.[18]

Previously, we modified the murine L-cell line GLUTag to exhibit stable expression of human insulin[24] such that large numbers of cells secreting insulin in a uniform manner could be cultured and used in tissue-engineered pancreatic substitutes based on such cells. Subsequently, a disk-shaped construct, in which an inner cell-containing disk was encapsulated in an outer layer of cell-free agarose, was fabricated and characterized.[25,26] *In vitro*, these constructs were found to support the steady function of GLUTag-INS cells for an extended period of time, with nearly steady levels of total DNA content, glucose consumption rates, and basal insulin secretion rates over 4 weeks of culture. Only constructs that permitted initial cell growth on small intestinal submucosa (SIS) before encapsulation, however, were able to maintain the induced secretion function of GLUTag-INS cells.[25,26] As such, these constructs were evaluated *in vivo* by implantation into diabetic mice. This article reports on results of this *in vivo* study, along with results from implantation of the same types of construct-containing parental GLUTag or murine insulinoma βTC-tet cells.

Methods

Small intestinal submucosa-containing constructs of disk geometry were fabricated as described previously.[25,26] Briefly, pieces of SIS (14 mm in diameter, Cook Biotech, West Lafayette, IN) were seeded with cells and cultured for 1 week prior to encapsulation in 2% SeaPlaque agarose. Final dimensions of the constructs were 15 mm in diameter and 2 mm in height (or 350 μl in volume). Constructs were seeded with recombinant GLUTag-INS, parental GLUTag, or insulinoma βTC-tet cells with approximately 3×10^6 cells per construct or contained no cells (sham treated) in the case of healthy and diabetic controls. Prior to implantation, insulin secretion from GLUTag-INS- and βTC-tet-containing constructs was evaluated by overnight incubation in culture medium, and insulin content in the medium samples was assayed by radioimmunoassay (RIA; Millipore, Billerica, MA).

All animal procedures were approved by the Georgia Institute of Technology Institutional Animal Care and Use Committee. Diabetes was induced in normal, C57BL/6J mice by giving a single injection of 200 mg/kg STZ while mice were under isoflurane anesthesia and was defined as those mice with two consecutive nonfasting blood glucose levels of more than 250 mg/dl. The diabetic state was determined by blood glucose monitoring with the TrueTrack Smart System® (Home Diagnostics, Inc., Ft. Lauderdale, FL) using blood collected at the tail tip site.

Four days postimplantation, mice were euthanized by CO_2 inhalation, and approximately 1 ml of blood was obtained by cardiac puncture. Blood was allowed to clot at room temperature and was then centrifuged at 1600 *g* at 4°C to isolate the plasma. Plasma was frozen at –80°C until assayed by the human ultrasensitive RIA and/or the rat insulin RIA (Millipore). While analysis on both kits would have been preferred, the limited serum volumes from mice prevented measurements of both mouse and human insulin in serum samples. Explanted constructs were placed in complete culture medium and returned to incubator conditions overnight to assess insulin secretion postexplantation. Insulin concentrations in medium samples taken preimplantation

and postexplantation were determined using the human RIA for GLUTag-INS-containing constructs or the rat RIA for βTC-tet-containing constructs. One-way analysis of variance was performed using MINITAB 14; $p < 0.05$ indicated significant difference.

Results

Although there was no effect of GLUTag-INS- or βTC-tet-containing constructs on body weight or blood glucose levels, human and murine insulin were detected in the plasma of GLUTag-INS- and βTC-tet-treated mice, respectively (**Figure 1**). This level was significantly higher than the level detected in mice treated with cell-free constructs. The human insulin level measured in healthy mice in **Figure 1B** is apparently due to the cross-reactivity of murine insulin with the human RIA and, as it was similar to the level detected in sham-treated mice, was quite low. Notably, mice treated with murine βTC-tet cells had significantly elevated plasma levels of murine insulin compared to all other groups, including healthy mice (**Figure 1A**). The range of murine insulin in healthy mice was markedly large. This high variability was likely the result of free access to food that the mice had and the lack of a fasting period prior to blood sampling.

In order to quantify the insulin secretory function of the constructs both preimplantation and postexplantation, constructs were incubated with complete culture medium overnight and the accumulated insulin was assayed. These values are compared in **Table 1**. While insulin was detected at both time points, the amount measured following explantation was significantly lower than preimplantation values for both GLUTag-INS- and βTC-tet-containing constructs.

Discussion

The disk-shaped SIS/agarose constructs described here, although unable to restore normoglycemia in the STZ mouse model, did deliver insulin *in vivo* as evidenced by the elevated plasma insulin levels and the retention of some secretory function postexplantation. The magnitude of the decline in insulin secretory function

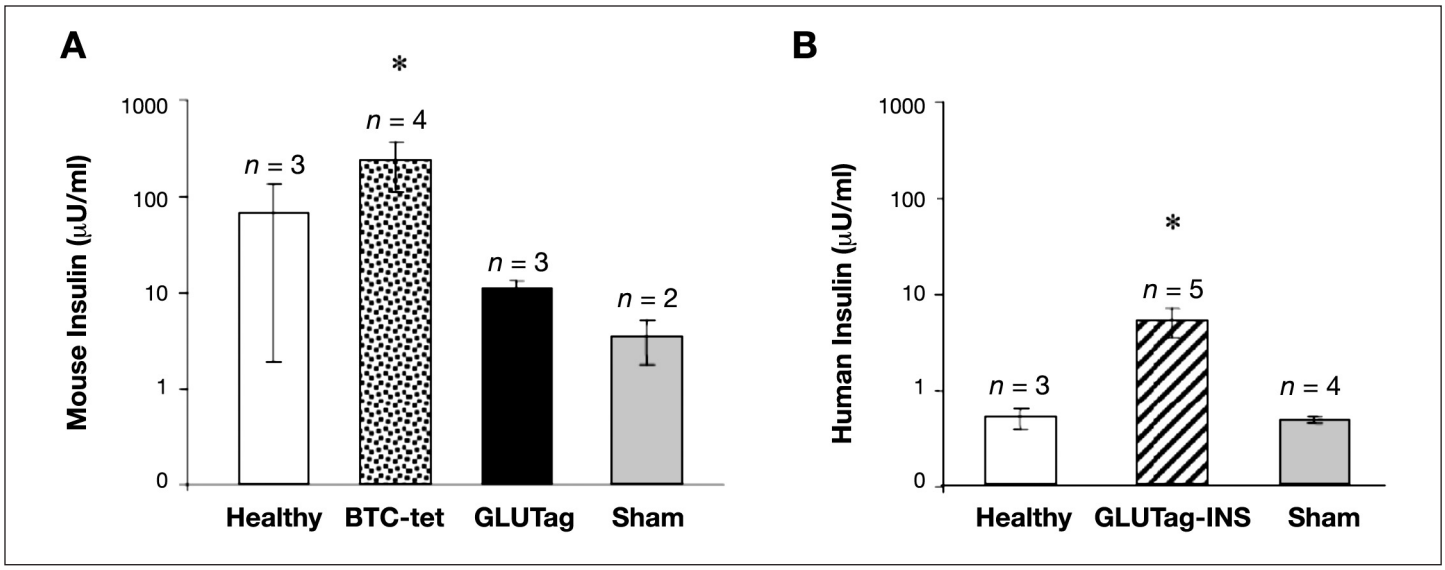

Figure 1. Blood plasma levels of murine **(A)** and human **(B)** insulin in mice implanted with disk-shaped constructs. The normal value in healthy mice detected with each kit is the far left bar (open) in each graph, whereas the far right bar (gray) is the value detected for sham-treated diabetic mice. Insulin concentration (μU/ml) is shown on a log scale. *Different than all other groups in graph (one-way analysis of variance, $p < 0.05$).

Table 1.
Insulin Secretion Rates from Constructs Measured before Implantation and after Explantation during Overnight Incubation in Complete Medium

	Human insulin from GLUTag-INS cell-based constructs	Murine insulin from βTC-tet cell-based constructs
Preimplantation	216.7 ± 13.6 μU/(construct · day)	65.2 ± 11.9 mU/(construct · day)
Postexplantation	56.2 ± 7.9 μU/(construct · day)	2.0 ± 1.8 mU/(construct · day)
Percentage of preimplantation	25.9 ± 3.4%	2.8 ± 2.3%

for the constructs was surprising, however, and possible contributing factors include the immune response to exposed cells in compromised constructs and the diffusion-limited supply of oxygen toward the encapsulated cells. For mice implanted with constructs containing βTC-tet or GLUTag-INS cells, a straightforward explanation is available for the presence of murine or human insulin, respectively, in plasma samples, as these constructs demonstrated insulin secretion both before implantation and after retrieval. Interestingly, although the plasma insulin detected in diabetic mice implanted with βTC-tet-containing constructs was significantly higher than the plasma insulin of healthy controls, blood glucose concentrations were not returned to normal values. A possible explanation for this rests with the physiological changes that take place during extended periods of hyperglycemia, chiefly the increase in peripheral insulin resistance. It has been reported that hyperglycemia in STZ-induced diabetes leads to progressive insulin resistance of the peripheral tissue.[27] Changes that occur following hyperglycemia include an impairment of GLUT4 translocation in skeletal muscle[28] and an inhibition in glycogen synthase in adipocytes.[29] Thus, glycemic regulation appears to depend on the initial state of the animal. Whereas one level of insulin results in a certain blood glucose level prior to the onset of diabetes, a different amount of insulin may be required to achieve the same blood glucose level after the onset of diabetes. This means that curing diabetes by a cell substitute is further complicated by the path that glycemic regulation has taken.

Although the rise of murine insulin in mice treated with parental GLUTag cells compared to negative controls was not statistically significant, it is possible that a larger study might have elucidated a difference. This is because GLP-1 was still secreted from the construct, and GLP-1 is known to potentiate the secretion of insulin from β cells in the presence of glucose.[30] Known actions of GLP-1 also include restoring glucose competence in glucose-resistant β cells; stimulating insulin gene expression and biosynthesis; and promoting cell proliferation, survival, and neogenesis.[31] Because β cells in the animals used were specifically ablated by STZ, the precise action of GLP-1 is more complicated. It is possible that (1) some percentage of pancreatic β cells evaded harm from STZ administration and GLP-1 served to potentiate their insulin secretion, (2) damaged (but not destroyed) β cells were able to recover some function following GLP-1 action, (3) GLP-1 worked to increase β-cell mass by regeneration, or (4) a combination of these mechanisms was at work in mice implanted with GLP-1-secreting cells.

Based on the aforementioned observations, a single type of non-β cell, engineered genetically for insulin secretion, is unlikely to reproduce the complex biphasic response of normal β cells with high fidelity. From the two cell types considered, enteroendocrine cells expressing recombinant insulin appear capable of providing the acute phase of secretion, as they package insulin—and endogenous GLP-1—in secretory granules, which are exocytosed rapidly upon metabolic stimulation.[24,32] Hepatocytes do not possess a system of secretory granules, which accumulates presynthesized insulin, so when insulin is expressed in these cells under transcriptional regulation, insulin secretion exhibits a slower but more sustained response to secretion agonists.[18,19] Hence, it is possible that a combination of these two cell types, in the appropriate ratio, reproduces the biphasic secretory response of normal β cells better than either cell type alone. This concept is shown schematically in **Figure 2**. Supplementing the insulin secretion from recombinant L cells with that from recombinant hepatocytes offers the additional advantage of being easier to achieve the therapeutic levels of insulin needed to treat hyperglycemia.

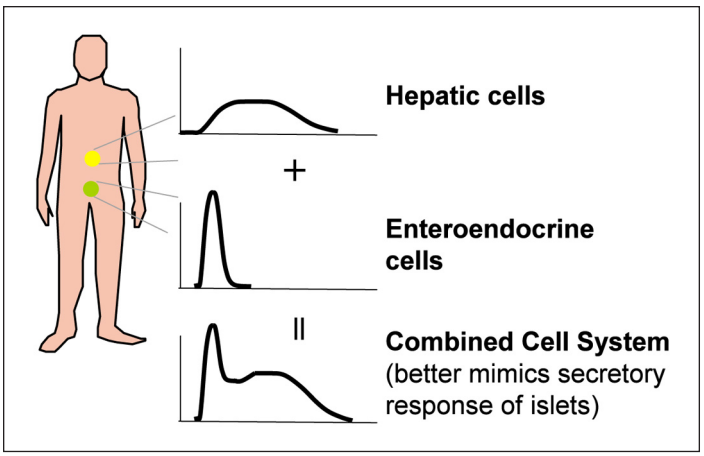

Figure 2. Insulin secretion dynamics in response to metabolic stimulation of hepatic cells expressing insulin under transcriptional regulation and of enteroendocrine cells, which accumulate insulin in secretory granules. A combination of the two cell types may mimic the secretory response of normal islets with higher fidelity than either cell type alone.

Conclusions

Cell-based therapies for IDD have the potential of providing a good regulation of blood glucose levels, thus reducing long-term complications. Use of human and animal islets is limited by reduced tissue availability and/or immune acceptance issues. Somatic non-β cells

engineered genetically for physiologically responsive insulin secretion may relax both of these limitations. Mouse enteroendocrine cells were engineered genetically for the stable expression of human insulin; when incorporated in a tissue construct and implanted in diabetic mice, human insulin was detected in the plasma of the animals, although normoglycemia was not restored. It is hypothesized that a combined system of hepatic cells expressing insulin under transcriptional regulation and of enteroendocrine cells, which release insulin via a regulated secretion pathway, may better reproduce the secretory function of normal islets than either cell type alone.

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