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Failure to Achieve Normal Metabolic Response in Non-Obese Diabetic Mice and Streptozotocin-Induced Diabetic Mice After Transplantation of Primary Murine Hepatocytes Electroporated With the Human Proinsulin Gene (p3MTChins)

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Abstract

Background—A recent study by Chen et al described a therapy for diabetes that involved electroporation of primary hepatocytes with human proinsulin cDNA, p3MTChins. Intrahepatic transplantation of treated hepatocytes into streptozotocin (STZ) murine and porcine models led to euglycemia, weight maintenance, and normal insulin production. We tested the repeatability of their basic experiments and transplantation technique and expanded the study to include an autoimmune model.

Methods—Hepatocytes were isolated from B6 mice, electroporated with p3MTChins, and glucose-challenged or were injected into hepatic or spleen parenchyma of STZ-diabetic B6 and non-obese diabetic mice. Outcomes included survival, serum glucose levels, insulin, and c-peptide release. Untransfected primary hepatocytes and mice transplanted with these cells served as controls.

Results—p3MTChins-hepatocytes secreted insulin during glucose challenge, but glucose levels did not change with increasing glucose concentrations. Direct hepatic injection led to high mortality rates. Mice that underwent intrasplenic injection survived for >50 days (control = 4 days) and had a mild but stable improvement in hyperglycemia. C-peptide in both mouse models was detectable but eventually declined to baseline in the non-obese diabetic mice.

Conclusions—Hepatocytes can be transfected with p3MTChins to produce human insulin but may lack the proper glucose-sensing or complex storage and secretion capabilities that allow for a finely tuned dynamic insulin response. Treatment is subtherapeutic, and p3MTChins-hepatocyte function may not endure in an autoimmune model. Without successful preliminary findings, cell therapy involving electroporation of p3MTChins does not appear to be practical as a therapy for diabetes and may not be a strategy to pursue at this time.

THE EXPRESSION of an insulin transgene from a glucose-responsive promoter in human hepatocytes has been described to be a potential strategy for the treatment of insulin-dependent diabetes. This method has known advantages over other cell replacement therapies, prompting efforts to examine this method's efficacy and stability in vivo [1,2].

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Freshly isolated hepatocytes are readily electroporated with a non-viral vector [3,4], efficiently engraft in structurally normal liver, and have been shown to have fewer inflammatory and tumorigenic properties compared with transfection with adenovirus, retroviral vectors [5–7], and pluripotent stem cells [8–11]. In addition, transplantation of autologous hepatocytes prevents immune rejection, overcomes the adverse effects of immunosuppressive regimens, and is more available than allogeneic β-cell transplantation [12,13]. Insulin-expressing hepatocytes also lack β-cell epitopes, protecting them from autoimmune destruction inherent in insulin-dependent diabetes [14].

A recent study by Chen et al [1,2] described the therapeutic effectiveness of this strategy in murine and porcine models. By delivering a human proinsulin cDNA plasmid construct (p3MTChins, National Cancer Centre, Singapore) to primary hepatocytes through ex vivo electroporation, the authors were able to achieve durable treatment of streptozotocin (STZ)-induced type 1 diabetes mellitus (T1DM). They demonstrated physiologically productive insulin secretion with rapid secretion kinetics, weight restoration, and ultimately, long-term euglycemic control. However, it is still unclear how transfected hepatocytes can robustly function as β-cell surrogates. Unlike the β-cell, p3MTChinshepatocytes synthesize insulin primarily through transcription and cannot form an insulin response to glucose as rapidly in the acute glucose setting [15]. Without storage granules and a regulated secretory pathway, hepatocytes also appear to be ill-equipped for the dynamic requirements to achieve euglycemic control [16].

The primary purpose of this study is to reproduce the principal findings of Chen et al. We are particularly interested in examining p3MTChins-hepatocyte response to in vitro glucose challenge, feasibility of direct hepatic injection, and level of secretion of insulin and c-peptide in both STZ-diabetic and non-obese diabetic (NOD) mice. By expanding the diabetic model to include the NOD strain, we hope to determine whether transfected hepatocytes are protected from the autoimmune process responsible for clinical T1DM.

METHODS

All materials and methods used in this study were approved by and in compliance with the Institutional Animal Care and Use Committee standards (AN085365-01).

Isolation of Primary Murine Hepatocytes

Male mice of the C57B6 strain (8–12 weeks old) were used as hepatocyte donors. Hepatocytes were isolated by means of portal vein collagenase perfusion with a 24-gauge catheter (Terumo Medical Corporation, Somerset, NJ, United States). Gentle dissociation with scissors was used to separate the parenchymal cells from the liver capsule before filtration through a 70-μm filter (Fischer Medical Technologies, Broomfield, Colo, United States). Gravity filtration on ice yielded a relatively pure single-cell suspension of hepatocytes in Dulbecco’s modified Eagle’s medium (DMEM; Gibco Life Technologies, Grand Island, NY, United States) with 10% fetal bovine serum.

Ex Vivo Gene Transfer

Transfection was performed by means of the Nucleofector electroporation system with equal volume mixtures of solutions No. 3551 and No. 3541 with the use of program T28 (Amaxa Bio-systems, Koln, Germany). Eight milligrams of endotoxin-free plasmid DNA (p3MTChins) was added to each cuvette in 0.2 mL of electroporation solution. Electroporation was performed in a sterile cuvette with a single 1400-V pulse for 70 ms, followed by a single pulse of 160 V for 37 ms.

Induction of Diabetes With STZ

Daily intraperitoneal injections of STZ (100 mg/kg body wt/day) were administered over a period of 4 days. Mice were considered diabetic if fasting blood sugar levels exceeded 260 mg/dL by day 5, determined by means of a glucometer and glucose test strips on tail vein blood samples.

Hepatocyte Transplantation

Hepatocytes were transfected with p3MTChins on day 6 after STZ injection as described above. Either the right medial and lateral lobes of the liver or distal spleen were visualized by means of a 5-mm right upper quadrant incision or a 3-mm left flank incision. Hepatocytes (1×10^6) in single-cell suspension in DMEM (Gibco) with 10% fetal bovine serum were delivered by direct interstitial injection into either the right lateral and right medial lobes of the liver or the distal spleen with the use of a 31-gauge syringe (Fischer). After the splenic injection, 3 mm of the distal spleen was ligated with 4–0 silk suture (Fischer) to prevent back-bleeding. Incisions were closed with running 4–0 vicryl suture (Fischer).

Induction and Measurement of Gene Expression In Vitro

Freshly isolated primary hepatocytes transfected with p3MTChins were plated with the use of collagen-coated 35-mm dishes in 25 mmol/L of glucose DMEM supplemented with fetal calf serum, penicillin, and streptomycin. The plates were incubated in 5% CO_2 at 37°C for 16 hours with media changed after 5 hours and replaced with fresh DMEM containing 2 glucose concentrations (30 mg/mL, 300 mg/mL). Supernatants were assayed for human insulin content with the use of the Immulite Immunometric assay (Diagnostic Products, Los Angeles, Calif, United States) after 24 hours. As an internal control, human c-peptide was also measured with the use of the c-peptide enzyme-linked immunoassay kit (ALPCO, Salem, NH, United States).

Measurement of Glucose and Insulin Expression In Vivo

Fasting glucose tests were performed on diabetic and control mice with the use of tail vein blood samples, a glucometer, and glucose test strips. As described above, tail vein samples were assessed for human insulin and human c-peptide content.

RESULTS

Fresh hepatocytes were isolated with approximately 95% viability and were readily electroporated with GFP-p3MTChins (figure not shown) with 50–60% efficiency. Glucose challenge of plated hepatocytes at low (30 mg/dL) and high (300 mg/dL) concentrations at 1 and 60 minutes demonstrated functional insulin secretion of similar levels in both glucose environments (Fig 1). Hepatocyte viability was estimated to be 95% after glucose challenge. STZ-diabetic B6 mice with p3MTChins-hepatocytes delivered through direct hepatic injection remained hyperglycemic and survived for no more than 5 days, similar to control (Fig 2). The STZ-diabetic mice that received p3MTChins-hepatocytes through intrasplenic injection had improved hyperglycemia compared with control but remained mildly hyperglycemic for more than >55 days (Fig 3A). This group of mice also had elevated and stable blood c-peptide levels for >55 days, as well as extended survival of >45 days compared with control (Fig 3B). In the NOD recipient, serum glucose nearly normalized (Fig 4A) as c-peptide levels peaked (Fig 4B). However, c-peptide eventually declined over a period of 62 days and correlated with an increase in serum glucose. Survival was extended in the NOD recipient compared with the control NOD mouse by 47 days.

DISCUSSION

In our experience, p3MTChins-transfected hepatocytes are a poor physiologic surrogate for β-cells. Although they have glucose sensing capacity and glucose-mediated transcription [17], they are unable to rapidly synthesize insulin through translation and release insulin through secretory granules [1]. As demonstrated by glucose challenge, both high and low glucose environments induced brisk secretion of insulin from hepatocytes in equal amounts. Our results differ from that of Chen et al, in which a high-glucose environment induced a rapid 4-fold increase in insulin production compared with a low-glucose environment. The differences in our findings are unclear because the same vector, number of hepatocytes, and similar glucose concentrations were used. There may be some element of cell lysis that caused the release of insulin into extracellular media. However, with such an acute exposure to glucose and in the absence of insulin storage granules, this is unlikely.

After immunostaining recipient liver, Chen et al had recognized that direct hepatic injection led to engraftment that was more evenly distributed, integrated into liver lobules, and into fewer extrahepatic areas when compared with delivery through the abdominal mesentery. We were unable to perform direct hepatic injection without high postoperative mortality rates and no measureable metabolic response from the transfected cells. Direct injection into the hepatic interstitium appeared to cause significant trauma despite a minimal incision and use of a fine-gauge needle. We found that hepatocytes were easily implanted into the distal spleen parenchyma and that mice had decreased mortality rates, stable weight maintenance, functional insulin secretion, and improved hyperglycemia. Immunohistochemistry of recipient livers after intrasplenic injection would explain hepatocyte migration patterns and sites of engraftment in the liver.

NOD mice are genetically susceptible to development of autoimmune insulinitis and have thus have been used as a preclinical model for autoimmune diabetes. Though hepatocytes

lack the β-cell epitopes that are targeted in T1DM, general autoimmune processes and cascades may affect the hepatocytes directly, affect the synthesis and release of insulin, or complicate the delicate process of hepatocyte migration and implantation. Compared with STZ-diabetic mice, the NOD recipient had similar survival time, initial blood glucose levels, and initial c-peptide release. However, c-peptide levels eventually dropped as serum glucose rose, which suggests that the NOD recipient lost p3MTChinshepatocytes function over time, as opposed to recovered β-cell function. Immunohistochemical staining of the liver after c-peptide drop-off would help to characterize p3MTChins-hepatocyte dysfunction. Of course, our conclusions are limited by the use of a single NOD mouse and could benefit from additional subjects.

Despite recent success with developing and transplanting a potential β-cell surrogate through electroporation of a non-viral vector, we are encountering a number of fundamental issues that prohibit further investigation into this therapy. Euglycemia is achieved through a dynamic sensory/response system–β-cells are rapidly dynamic and able to respond to acute changes in serum glucose in vitro and in vivo. There remain difficulties with the transfection and transplantation processes as well: p3MTChins is transfected with relatively low efficiency, and hepatocytes are fragile in incubation and in transplantation. They are difficult to transplant through direct hepatic injection, and their function may be susceptible to autoimmune processes. There are multiple factors that prevent p3MTChins-hepatocytes from becoming a true surrogate β-cell for therapy. Without a durable metabolic response from p3MTChins-hepatocytes in culture and in vivo, we believe that further investigation into this strategy for the treatment of T1DM is not warranted at this time.

Acknowledgments

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REFERENCES

[1]. Chen N, Sivalingam J, Tan S, et al. Plasmid-electroporated primary hepatocytes acquire quasi-physiological secretion of human insulin and restore euglycemia in diabetic mice. Gene Ther. 2005; 12:655–67. [PubMed: 15703765]

[2]. Chen N, Wong J, Kee I, et al. Nonvirally modified autologous primary hepatocytes correct diabetes and prevent target organ injury in a large preclinical model. PLoS One. 2008; 3:e1734. [PubMed: 18320053]

[3]. Kolodka TM, Finegold M, Moss L, et al. Gene therapy for diabetes mellitus in rats by hepatic expression of insulin. Proc Natl Acad Sci U S A. 1995; 92:3293–7. [PubMed: 7724555]

[4]. Thulé PM, Liu JM. Regulated hepatic insulin gene therapy of STZ-diabetic rats. Gene Ther. 2000; 7:1744–52. [PubMed: 11083496]

[5]. Liu Q, Muruve DA. Molecular basis of the inflammatory response to adenovirus vectors. Gene Ther. 2003; 10:935–40. [PubMed: 12756413]

[6]. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science. 2003; 302:415–9. [PubMed: 14564000]

[7]. Yeechor V, Chan L. Gene therapy progress and prospects: gene therapy for diabetes mellitus. Gene Ther. 2005; 12:101–7. [PubMed: 15496957]

[8]. Serup P. Embryonic stem cell-based diabetes therapy: a long road to travel. Diabetologia. 2006; 49:2537–40. [PubMed: 17019597]

[9]. Fujikawa T, Oh S, Pi L, et al. Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells. Am J Pathol. 2005; 166:1781–91. [PubMed: 15920163]

[10]. Wang Y, Huso D, Harrington J, et al. Outgrowth of a transformed cell population derived from normal human BM mesenchymal stem cell culture. Cytotherapy. 2005; 7:509–19. [PubMed: 16306013]

[11]. Miura M, Miura Y, Padilla H, et al. Accumulated chromosomal instability in murine bone marrow mesenchymal stem cells leads to malignant transformation. Stem Cells. 2006; 24:1095–103. [PubMed: 16282438]

[12]. Sapir T, Shternhall K, Meivar-Levy I, et al. Cell-replacement therapy for diabetes: generating functional insulin-producing tissue from adult human liver cells. Proc Natl Acad Sci U S A. 2005; 102:7964–9. [PubMed: 15899968]

[13]. Balamurugan AN, Bottino R, Giannoukakis N, et al. Prospective and challenges of islet transplantation for the therapy of autoimmune diabetes. Pancreas. 2006; 32:231–43. [PubMed: 16628077]

[14]. Tabiin MT, White CP, Morahan G, et al. Insulin expressing hepatocytes not destroyed in transgenic NOD mice. J Autoimmune Dis. 2004; 1:3. [PubMed: 15679918]

[15]. Itoh N, Okamoto H. Translational control of proinsulin synthesis by glucose. Nature. 1980; 283:100–2. [PubMed: 6985712]

[16]. Halban PA, Kahn SE, Lernmark A, Rhodes CJ. Cell therapy for type 2 diabetes: is it desirable and can we get it? Diabetes Obes Metab. 2008; 10(Suppl 4):205–11. [PubMed: 18834449]

[17]. Alam T, Sollinger H. Glucose-regulated insulin production in hepatocytes. Transplantation. 2002; 74:1781–7. [PubMed: 12499898]

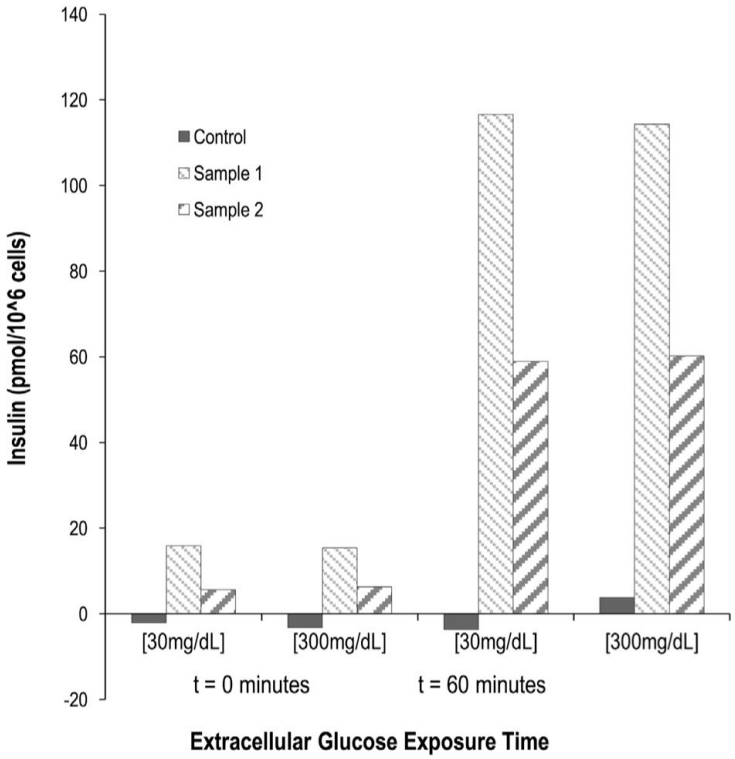

Fig 1.

Glucose challenge of B6 human proinsulin cDNA plasmid construct (p3MTChins)-hepatocytes.

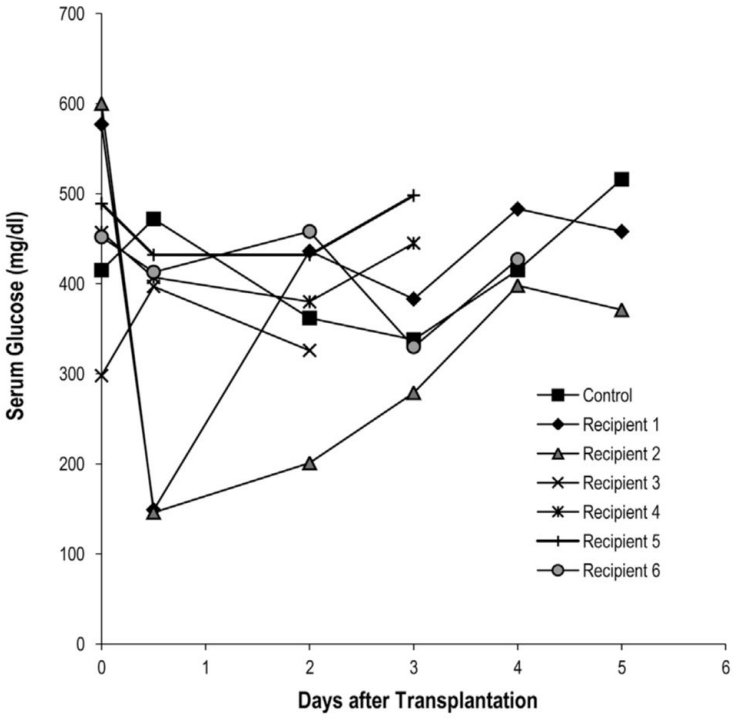

Fig 2.

Serum glucose levels in streptozotocin-diabetic mice after direct hepatic injection of human proinsulin cDNA plasmid construct (p3MTChins). All mice died by postoperative day 5.

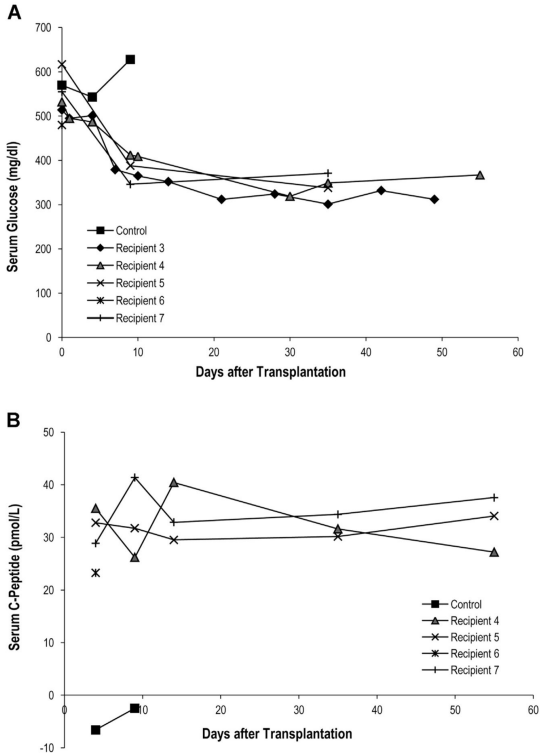

Fig 3.

Streptozotocin-diabetic B6 mice after intrasplenic injection. **(A)** Serum glucose levels; **(B)** serum c-peptide levels. Recipient 6 died soon after the procedure. Recipients 3, 4, 5, and 7 were euthanized on postoperative day 55.

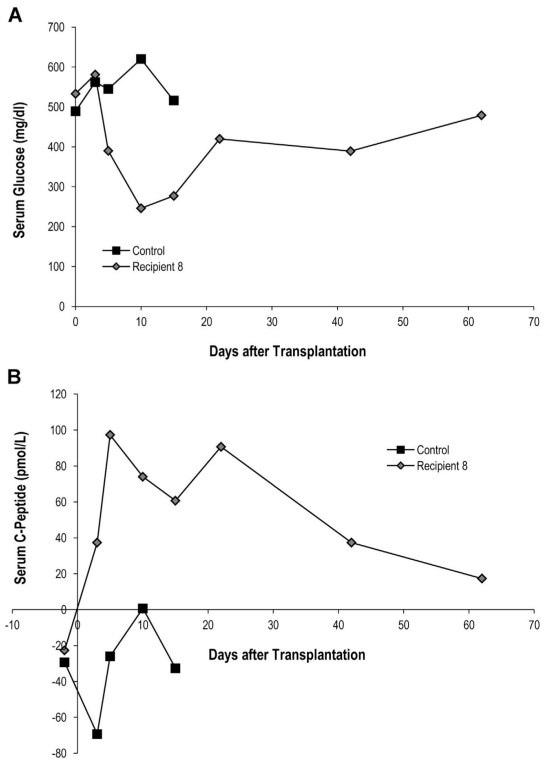

Fig 4.

Non-obese diabetic mice after intrasplenic injection. **(A)** Serum glucose levels; **(B)** serum c-peptide levels. Recipient 8 died on postoperative day 68.