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Research Paper

Normalization of glucose post-transplantation into diabetic rats of pig pancreatic primordia preserved in vitro

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Abbreviations: E, embryonic day; ES, embryonic stem cell; NCAM, neural cell adhesion molecule; Rip, rat insulin promoter; SLA, swine leukocyte antigen; STZ, streptozotocin; Tag, T antigen

Key words: beta cell, diabetes mellitus, transplantation, xenotransplantation

Embryonic day (E) 28 (E28) pig pancreatic primordia transplanted into the mesentery of non-immunosuppressed streptozotocin (STZ)-diabetic Lewis rats normalize levels of circulating glucose within 2–4 weeks. Exocrine tissue does not differentiate after transplantation of pancreatic primordia. Rather individual endocrine (beta) cells engraft within the mesentery.

To determine whether transplanted pig pancreatic primordia engraft, differentiate and function in rat hosts after preservation in vitro, we implanted pig pancreatic primordia into STZ-diabetic rats either directly or after 24 hours of suspension in ice-cold University of Wisconsin (UW) preservation solution with added growth factors. Here we show engraftment in mesentery and mesenteric lymph nodes and normalization of glucose levels in STZ-diabetic rat hosts following transplantation of preserved E28 pig pancreatic primordia comparable to glucose normalization after transplantation of non-preserved E28 pancreatic primordia.

Introduction

One strategy to overcome the limited potential for growth/division of mature islet cells is to transplant fetal pancreatic tissues (pancreatic primordia) that have a capacity for beta cell expansion post-implantation.^{1,2} Their potential for expansion sufficient to render a diabetic host euglycemic is reflected by normalization of glucose levels in a diabetic rat post-transplantation of a single primordium.³ To establish the feasibility of pig-to-rodent xenotransplantation of pancreatic primordia, we implanted pancreatic primordia from embryonic day (E) 28 (E28) pig embryos into streptozotocin-diabetic adult Lewis rats. Remarkably if transplanted within a developmental 'window' prior to E35 (E28 works best), pig pancreatic primordia engraft post-implantation into

non-immunosuppressed immunocompetent diabetic rat hosts.^{4,5} Glucose tolerance in streptozotocin-diabetes in rats is normalized permanently by the physiological secretion of porcine insulin from transplanted pig pancreatic primordia.⁵ We have shown that glucose tolerance can be normalized post-transplantation of E28 pig pancreatic primordia into non-immunosuppressed ZDF rats, a model for insulin-resistant type 2 diabetes in humans.⁶ Recently we have shown that E28 pig pancreatic primordia engraft long-term (~2 years) in mesenteric lymph nodes following transplantation into the mesentery of STZ-diabetic rhesus macaques. Exogenous insulin requirements are reduced significantly within four weeks after transplantation of primordia. Following transplantation porcine insulin, but not rhesus macaque insulin can be detected in circulation. These data support potential clinical applicability for transplantation of pig pancreatic primordia into diabetic humans.⁷

Theoretically, if employed for clinical use in humans, pancreatic primordia could be harvested immediately prior to implantation. However, practically it would be best if pancreatic primordia could be stored in vitro for a period of time prior to transplantation. To determine whether E28 pig pancreatic primordia can be stored in vitro prior to transplantation, we transplanted pancreatic primordia from E28 pig embryos into the mesentery of non-immunosuppressed streptozotocin-diabetic (host) rats either directly or after 24 hours of preservation in vitro. Here we show that preserved E28 pig pancreatic primordia engraft and normalize levels of glucose in STZ-diabetic rat hosts.

Results

We compared levels of glucose in each of four groups of rats: (1) Control, non-diabetic rats (n = 4); (2) Non-immunosuppressed diabetic Lewis rats into which five E28 pig pancreatic primordia were transplanted within 45 minutes of isolation (TX) (n = 4); (3) Non-immunosuppressed diabetic Lewis rats that underwent sham surgery (Diabetic) (n = 4); or (4) Non-immunosuppressed diabetic Lewis rats into which five E28 pig pancreatic primordia that had been preserved in vitro for 24 hours were transplanted (TX PRESERVED) (n = 3).

Following administration of streptozotocin and prior to transplantation or sham surgery (Week 0) rats in Diabetes; TX and

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TX-PRESERVED groups were grossly hyperglycemic. In contrast, on weeks 4–6, glucose levels were no different between rats in the control group (Control); rats into which five E28 pig pancreatic primordia had been transplanted 4–6 weeks before (TX); or rats into which five preserved E28 pig pancreatic primordia had been transplanted 4–6 weeks before (TX PRESERVED). As before,⁴ levels of glucose were not reduced post-sham transplantation (Fig. 1). We have shown previously that levels of glucose are normalized for as long as 20 months post-transplantation of E28 pig pancreatic primordia into formerly diabetic rats.⁵ At one year post-transplantation, levels of glucose in the TX-PRESERVED group were 105 ± 4 mg/dl ($n = 3$), no different than levels in the four control rats (121 ± 5 mg/dl). Three of the four sham-operated (Diabetic) rats did not survive one year. One was dead by 14 weeks post-surgery (glucose measured at 12 weeks was 316 mg/dl). The second was dead by 21 weeks post-surgery (glucose measured at 20 weeks was 228 mg/dl). The third was dead by 41 weeks post-surgery (glucose measured at 40 weeks was 550 mg/dl). The survivor was hyperglycemic at 52 weeks post-sham surgery (glucose was 434 mg/dl).

Shown in Figure 2 are tissue sections from formerly diabetic rats into which preserved pig pancreatic primordia had been transplanted one year previously. Cells expressing porcine proinsulin mRNA that stain with use of the antisense probe (Fig. 2B, D, F and H), but not the sense probe (Fig. 2A, C, E and G) are present in medullary sinus of mesenteric lymph nodes (Fig. 2A–F) and in mesentery (Fig. 2G and H). Rare porcine-proinsulin expressing cells are detectable in liver (not shown). Kidney, pancreas and spleen are negative for porcine proinsulin-expressing cells.

Cells in medullary sinus of mesenteric lymph nodes and mesentery that stain positive for insulin are shown in Figure 3. Sections B and D show insulin-positive cells (red). Control stains are shown in Figure 3A and C.

Discussion

The insufficient supply of tissue, loss post-transplantation and limited potential for expansion of beta cells restricts the utility of islet allotransplantation.⁹ A way to address the supply problem is to transplant pig tissue.¹⁰ A theoretical way to overcome the expansion problem is to transplant embryonic pancreas that can undergo expansion in situ.^{1,2}

In previous studies, we implanted whole E12.5 Lewis rat pancreatic primordia^{4,11} or E28–29 Yorkshire pig pancreatic primordia^{4,5} into the mesentery of adult streptozotocin-diabetic Lewis rats. Control of diabetes is achieved within 2–4 weeks following transplantation and the abnormal pattern of weight gain in diabetic animals restored to normal post-transplantation. Consistent with the findings of others,^{1–3} exocrine tissue does not differentiate. Rather, following islet transplantation in rats, the pancreatic primordia differentiate into novel structures consisting of islets surrounded by stroma.^{4,11} Rather than islets surrounded by stroma, only isolated endocrine cells engraft within the rat mesentery at 20 weeks post-xenotransplantation of E28 or E29 pig pancreatic primordia.^{4,5} If obtained from E28 or E29 embryos pig pancreatic primordia engraft post-implantation into STZ diabetic Lewis rat hosts without the need for immunosuppression. In contrast, pancreatic primordia obtained from E35 embryos are rejected and glucose levels remain elevated.⁵ E28 pig pancreatic primordia engraft long-term in non-immunosup-

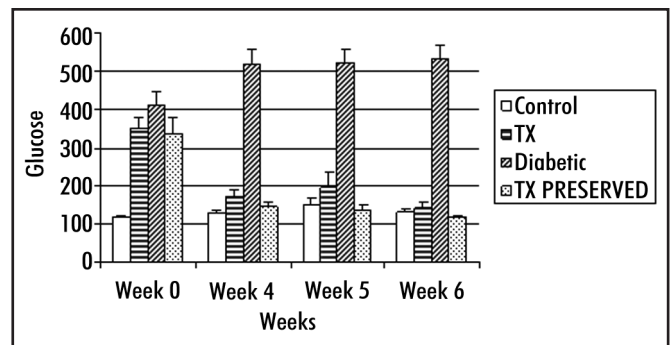


Figure 1. Levels of glucose in non-diabetic (Control) rats ($n = 4$) and in streptozotocin-diabetic rats into which 5 E28 pig pancreatic primordia had been transplanted (TX) ($n = 4$); or in which sham surgery was performed (Diabetic) ($n = 4$) or into which 5 E28 pig pancreatic primordia that had been preserved in vitro for 24 hours had been transplanted (TX PRESERVED) ($n = 3$); following measurement of glucose at week 0. Data are expressed as mean \pm standard error of the mean.

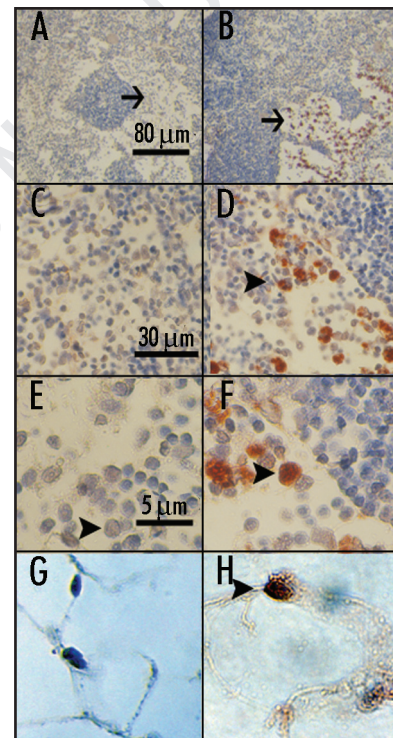


Figure 2. (A–F) Mesenteric lymph nodes (G and H) Mesentery originating from diabetic Lewis rats into which preserved E28 pig pancreatic primordia had been transplanted one year previously: In situ hybridization was performed using pig proinsulin sense (A, C, E and G) or antisense (B, D, F and H) probes. Arrow delineates medullary sinus in A and B. Arrowhead designates cells that stain positive for porcine proinsulin mRNA (D, F and H) and a negative-staining cell with similar morphology (E). Magnifications are shown in (A) for (A and B); in (C) for (C and D); and in (E) for (E–H).

pressed diabetic non-human primates (rhesus macaques) and reduce exogenous insulin requirements consistent with a potential for their use in diabetic humans.⁷ Although diabetes was not ‘cured’ in our rhesus macaques, it is possible that insulin requirements post-transplantation can be reduced further by transplanting more tissue at a given time or by repetitive transplantation or by enhancing beta cell

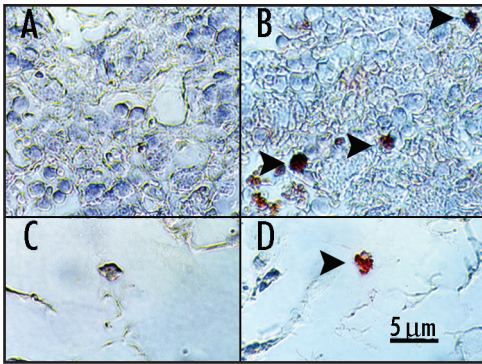


Figure 3. Photomicrographs of (A and B) rat mesenteric lymph nodes; (C and D) rat mesentery 1 year post-transplantation of preserved E28 pig pancreatic primordia. (A and C) Control serum-stained sections; (B and D) Anti-insulin antibody-stained sections. Arrowheads delineate endocrine cells (B and D). Magnification is shown in (D).

growth *in vivo* after transplantation. Future studies will be necessary to explore such possibilities in order that normalization of glucose tolerance in hosts reaches a threshold for clinical application.

While data generated in rhesus macaques will provide insight into the numbers of pig pancreatic primordia that might be required to correct diabetes in a human, and the time it will take post-transplantation for the primordia to produce sufficient insulin to render hosts euglycemic, only studies in humans, if and when conducted, can provide definitive answers to these questions.

In the case of human allotransplantation, there is an unavoidable delay between the time of harvest from donors and the time of implantation into recipients. Prior to removal from the donor, human renal allografts are flushed with a preservation solution, often University of Wisconsin (UW) solution, and stored subsequently in ice-cold UW solution.^{8,12} Theoretically, pancreatic primordia used for transplantation could be harvested immediately prior to implantation. However, practically it would be best if, like most grafts, pancreatic primordia could be stored *in vitro* for a period of time prior to transplantation. The ability to store pancreatic primordia would permit their distribution to sites for transplantation, distant from the site of harvesting and would allow time to plan the transplant procedure. Here (Figs. 1–3) we demonstrate viability, as evidenced by normalization of glucose and engraftment in hosts, for preserved E28 pig pancreatic primordia transplanted into rats. Preservation for 24 hours appears to have no detrimental effects whatsoever.

Transplantation of embryonic pancreas to replace the function of a diseased organ^{1-7,11,13} (organogenesis of the endocrine pancreas) offers theoretical advantages relative to transplantation of either embryonic stem (ES) cells, or of fully differentiated (adult) pancreas or islets from adults or neonatal animals. Thus: (1) Unlike ES cells, pancreatic primordia obtained at the proper time during embryogenesis differentiate along defined organ committed lines. There is no requirement to steer differentiation and no risk of teratoma formation;¹³ (2) The growth potential of cells within embryonic pancreas is enhanced relative to those in terminally-differentiated adult pancreas or adult islets in that it is possible to restore glucose tolerance in a diabetic rat by transplanting a single pancreatic primordium within 2–4 weeks.³ Neonatal pig islets transplanted into pan-

atectomized immunosuppressed pigs normalize levels of glucose within a comparable time period post-transplantation (14 days).¹⁴ (3) Only endocrine tissue differentiates following transplantation of pancreatic primordia obtained sufficiently early during embryogenesis^{1-7,11} obviating problems of host tissue digestion by exocrine pancreatic components;³ and (4) The cellular immune response to transplanted embryonic pancreas obtained early during embryogenesis is attenuated relative to that directed against organs obtained at later times.¹⁵

During normal pancreatic organogenesis, individual endocrine cells first migrate away from primitive ducts prior to coalescing into islets. Migration and coalescence are guided by cell adhesion molecules.¹⁶ We speculate that the failure of individual pig endocrine cells to coalesce into islets post-transplantation of E28 pig pancreatic primordia into rats⁴⁻⁶ or non-human primates,⁷ may result from the absence of adhesion molecules in rat⁴⁻⁶ or primate⁷ interstitium that are recognized by pig endocrine cells. In contrast rat endocrine cells recognize mouse adhesion molecules.¹¹ One explanation for our ability to transplant E28 pig pancreatic primordia without immunosuppression into rats or rhesus macaques may be that the absence of both exocrine tissue and islets results in a pattern of antigen expression that is not recognized as foreign by the host.^{6,7} An alternative explanation is host tolerance on the basis of microchimerism or mixed chimerism as proposed by Abraham et al., to explain successful xenograftment of human pancreatic islet-derived progenitor cells in multiple tissues of non-immunosuppressed immunocompetent mice.¹⁷ Another is 'T cell paralysis'¹⁸ on the basis host exposure to antigen plus SLA II that is expressed on pig beta cells^{19,20} in the absence of second costimulatory signal.

Neural cell adhesion molecule (NCAM) is an important regulator of endocrine cell aggregation during islet development.²¹ Crnic et al. showed that loss of NCAM function causes the formation of lymph node metastasis in a transgenic model of pancreatic beta cell carcinogenesis (Rip1Tag2). Metastases were facilitated by upregulated pancreatic lymphangiogenesis possibly induced by disaggregation of endocrine cells.²² It may be that failure of endocrine cells to aggregate following transplantation of E28 pig pancreatic primordia in the mesentery of rats⁴⁻⁶ or rhesus macaques⁷ induces a comparable lymphangiogenesis that permits migration of beta cells to regional lymph nodes.

Methods

Isolation and transplantation of pancreatic primordia. Animal care followed Institutional Animal Care and Use Committee (IACUC) standards. At 28 days gestation, pregnant Yorkshire pigs (Oak Hill Genetics, Ewing IL) were intubated and anesthesia maintained by inhalation of isoflurane and O₂ to effect. The uterus was removed and the donor pig was then euthanized.⁴⁻⁶

Pancreatic primordia from E28 pig embryos were surgically isolated under a dissecting microscope.⁴⁻⁶ After isolation, primordia were placed immediately into ice-cold Dulbecco's modified Eagles Medium: Hams F12 (DMEM:HF12) containing iron-saturated transferrin (5 μg/ml), 25 nM prostaglandin E1 and, 5 μg/ml rhVEGF (Genentech Inc.) and 10⁻⁸ M rhHGF (Upstate Biotechnology, Lake Placid NY). After 45 minutes, five pancreatic primordia were implanted between layers of mesentery of 6–10 week old diabetic Lewis rat hosts in close proximity to blood vessels.⁴⁻⁶ Host rats received no immunosuppression. Other E28 primordia were suspended in 1 ml

ice-cold University of Wisconsin (UW) solution (ViaSpan, DuPont Pharmaceuticals) containing 10^{-9} M HGF; 10^{-9} M IGF I and 1 μ g/ml VEGF and stored for 24 hours at 4°C prior to transplantation. The growth factors and concentrations of growth factors added to the UW solution were chosen on the basis of our successful use of such a solution (UW + growth factors) to preserve renal primordia prior to transplantation into the mesentery of rats with reduced renal function.⁸ The composition of the UW solution differs in growth factor content from the ice-cold DMEM:HF12 in which non-preserved pancreatic primordia were suspended prior to implantation in this study and in previous studies.⁴⁻⁶ Other diabetic rats underwent sham surgery during which no primordia were implanted. Concurrently, other control rats were not rendered diabetic.

Measurement of glucose. Levels of glucose were measured using the Roche Diagnostic System/COBAS (Roche Diagnostic Systems, Somerville, NJ) in serum obtained (via tail vein) at 8 AM after an overnight fast.

Histology. At one year post-transplantation, tissue was removed from hosts. Tissues were fixed in 10% phosphate-buffered Formalin. The fixative was removed, tissues embedded in paraffin, sliced into 5 μ m sections and placed on glass slides in preparation for staining. Polyclonal rabbit anti-insulin antiserum was obtained from Accurate Chemicals (Westbury NY). Normal rabbit serum was substituted for the anti-serum in control stains. Sections were counterstained using hematoxylin.

Induction of diabetes mellitus and treatment with exogenous insulin. Diabetes mellitus was induced in rats by a single intraperitoneal injection of 65 mg/kg streptozotocin five days prior to transplantation of pancreatic anlagen. The diabetogenic response to streptozotocin varied from rat to rat. Rats were considered diabetic if their fasting blood glucose level was 225 mg/dl or above five days after streptozotocin administration, at which time pig pancreatic primordia were implanted. Maintenance of glucose levels below 200 mg/dl during the first 2–3 weeks post-transplantation is necessary to permit optimal differentiation, proliferation and function of transplanted E28 pig pancreatic primordia. Accordingly, rats transplanted with pig pancreatic primordia were treated with Humulin U ultralente insulin (Eli Lilly Indianapolis IN) for 2–3 weeks after implantation (1–4 units s.q bid) to keep levels of fasting glucose, measured every 3 days, at approximately 200 mg/dl. Administration of exogenous insulin was stopped in all rats after three weeks.⁵

Detection of insulin transcripts using in-situ hybridization. In situ hybridization was performed on sliced 5 μ m paraffin-embedded sliced tissue sections using a kit from GeneDetection (Bradenton FL) exactly as per the manufacturer's instructions (1 June 2001, Version 1.20). Sections were permeabilized with proteinase K [DAKO Cytomation Inc (Capintertia CA)]. We used mRNA In situ Hybridization Solution (DAKO) as pre-hybridization and hybridization buffer. We used horseradish peroxidase-mediated detection with Tyramide Signal Amplification [NEN/PerkinElmer (Wellesley MA) TSA Biotin System Catalog#NEL700]. Digoxin-labeled anti-sense probes (GeneDetection), that detect porcine, but not rat transcripts⁶ were complementary to nucleotides 230–264 of porcine preproinsulin cDNA: 5'-GGCGGAGAACCCTCAGGCAGGTGC-CGTGGAGCTGG-3' (Genbank: AY044828). All probes were labeled unless otherwise noted. A sense probe was used for control stains.

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