Islet cell engraftment and control of diabetes in rats after transplantation of pig pancreatic anlagen

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Rogers, Sharon A., Feng Chen, Mike Talcott, and Marc R. Hammerman. Islet cell engraftment and control of diabetes in rats after transplantation of pig pancreatic anlagen. Am J Physiol Endocrinol Metab 286: E502–E509, 2004.—The insufficient supply of tissue, loss posttransplantation, and limited potential for expansion of β-cells restrict the use of islet allotransplantation for diabetes. A way to overcome the supply and expansion problems is to xenotransplant embryonic tissue. We have shown that whole rat pancreatic anlagen transplanted into the omentum of rats, or xenotransplanted into costimulatory blocked mice, undergo growth and differentiate into islets surrounded by stroma without exocrine tissue. Isotransplants normalize glucose tolerance in diabetic hosts. Here, we show that embryonic day 29 porcine pancreas transplanted into the omentum of adult diabetic rats undergoes endocrine tissue differentiation over 20 wk and normalizes body weights and glucose tolerance. Unlike rat-to-rodent transplants, individual α- and β-cells engraft without a stromal component, and no immunosuppression is required for pig-to-rat transplants. Herein is described a novel means to effect the xenotransplantation of individual islet cells across a highly disparate barrier.

α-cell; β-cell; insulin; xenotransplantation

The use of pancreas or islet of Langerhans transplantation into humans as a means to treat type 1 diabetes mellitus is complicated by the need to immunosuppress hosts and limited by the scarcity of tissue to transplant (3, 26). In many respects, the pig is an ideal source of insulin-producing pancreatic cells for transplantation into diabetic humans in lieu of human tissue (11, 20, 23). Pigs are easy to breed and have large litters. Pigs are omnivorous and have a physiology similar to that of humans, no immunosuppression is required for these processes for application of this technology to treatment of type 1 diabetes in humans, no immunosuppression is required for these processes to occur in the immunocompetent Lewis rats.

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Iron-saturated transferrin (5/H9262) was employed because its overexpression in transgenic mice enhances cell proliferation and increases islet mass in vivo (10). VEGF was used to study angiogenesis on 3% agarose gels, and their identities were confirmed by sequencing the Washington University Core Protein and Nucleic Acid Chemistry Laboratory (Department of Molecular Pharmacology). For the specific amplification of pig insulin transcripts (GenBank: AY044828), the primers used were PlnExonF2: 5'-ctgtgtc-ccagccgca-3'; PlnExonR2: 5'-tccagcggactgcta-3'. For rat insulin 2 transcripts (GenBank: J00748), the primers used were RIns2IntronF1: 5'-ctgtctctggaattctatc-3'; RIns2ExonR2: 5'-tccaggttg-gcagctg-3'. For rat insulin 1 transcripts (GenBank: J00747), the primers used were RIns1ExonF1: 5'-ctccacggcacctgcctga-3'; RIns1ExonR2: 5'-tccagttgtggcacttgc-3'. The primers are intron spanning to eliminate the possibility of false amplification from genomic DNA, and they are species specific.

Measurement of insulin. Omentum (1 × 0.5 × 0.1 cm) was removed from rats and placed in DMEM containing 3 mM glucose at 37°C. After 1 h, glucose was added to the incubation to make the total concentration 20 mM, and 1 h later, two 100-μl aliquots were removed. Levels of porcine insulin in aliquots were measured using a porcine insulin-specific ELISA (catalog no. K6219; DAKO, Carpinteria, CA). The detection limit for porcine insulin added to media is 0.5 μU/ml media. Rat insulin standard (DAKO) was undetectable with use of the porcine specific assay at concentrations as high as 6.4 μU/ml.

Animal care. Animal care followed Institutional Animal Care and Use Committee standards (approval no. A3381-01). Statistics. Data are expressed as means ± SE and were analyzed using the Student-Newman-Keuls multiple comparison test. Differences for which P < 0.05 by two-tailed analysis were considered significant.

RESULTS

Use of growth factors for incubation of pancreatic anlagen. We have shown previously that normoglycemia in diabetic host Lewis rats can be restored within 30 days of transplantation of pancreatic anlagen obtained from E12.5 Lewis rat
embryos (21). To determine whether induction of normoglycemia in diabetic hosts can be accelerated if pancreatic anlagen are incubated with growth factors before implantation, we included VEGF and HGF in the DMEM-HF12 medium in which anlagen are stored before placement into hosts (see METHODS). Exactly as in previous studies that used nongrowth factor-incubated tissue (21), ten E12.5 Lewis rat pancreatic anlagen that were incubated with VEGF and HGF were implanted into each of three Lewis streptozotocin-diabetic rats. As before (21), this group was designated "transplant." Three other diabetic rats underwent sham surgery. As before (21), this group was designated "diabetic." Rats in the transplant and diabetic groups had levels of glucose measured during the next 40 wk in parallel with a group of three nondiabetic rats (as before, designated "control") (21).

In contrast to the case for hosts receiving nongrowth factor-incubated tissue, in which levels of glucose in the transplant group remained significantly elevated relative to the control group at 11 and 23 days postimplantation (21), levels of glucose in the transplant group were elevated significantly (P < 0.05) relative to the control group at 1 wk postimplantation and were no longer elevated (were normalized) at 2 wk posttransplantation and at later times (3–40 wk) in rats that received VEGF and HGF-treated tissue (Fig. 1). In contrast, the levels of glucose in the diabetic group were elevated significantly relative to controls at all times at which measurements were made.

Fig. 3. Photomicrographs of embryonic day 12.5 (E12.5) rat pancreatic anlagen 3 wk posttransplantation into a diabetic rat. A: hematoxylin and eosin (H&E)-stained section; B: control-stained section; C: anti-insulin antibody-stained section; D: combined Gomori-stained section. Islets are delineated by arrows. Data are representative of 3 experiments. Magnifications are shown in A.

Fig. 4. Levels of glucose (mg/dl) measured over time in each of 5 Control rats, Diabetic rats, and Transplant rats. Data are expressed as means ± SE.
The rate of weight gain over time is reduced in diabetic relative to nondiabetic rats (15). An early response to successful transplantation of rat pancreatic anlagen into diabetic rats is a restoration of the nondiabetic pattern of weight gain (15). To determine whether the normalization of glucose levels in our transplant group is reflected by a change in weight gain, rats in control, diabetic, and transplant groups were weighed over time. Before induction of diabetes in diabetic and transplant groups (week 0), and at 1 wk postinduction of diabetes (week 1), weights were slightly higher ($P < 0.05$) for the control group relative to diabetic and transplant groups. Weights were not different between diabetic and transplant groups at week 0 or week 1. At weeks 3, 6, 10, and 20 postinduction of diabetes, weights remained higher in the control group relative to the diabetic group. However, weights in control and transplant groups were no longer significantly different at 3, 6, 10, or 20 wk posttransplantation (Fig. 2). Therefore, normalization of glucose levels in the transplant group (Fig. 1) occurred in the context of a normalization of rat weight (Fig. 2).

A second set of transplantations was performed to obtain tissue for histological examination. H&E (Fig. 3A), control antibody (Fig. 3B), anti-insulin antibody (Fig. 3C), and Gomori-stained (Fig. 3D) sections of a developed growth factor-incubated E12.5 rat pancreatic anlage obtained 3 wk posttransplantation illustrate that, as was the case for nongrowth factor-treated anlagen (21), the tissue differentiates into structures consisting of islets of Langerhans (arrows) surrounded by stroma with no exocrine tissue present.

A third set of transplantations was performed to characterize the response of transplanted rats to a glucose challenge. To this end, $k$ values for glucose disappearance were measured at 6 wk posttransplantation in control, diabetic, and transplant groups. The values were $2.46 \pm 0.05$, $0.63 \pm 0.03$, and $2.81 \pm 0.40 \% / \text{min}$ in control, diabetic, and transplant groups, respectively ($n = 4$ rats per control and transplant groups, 3 rats in the diabetic group; control $> \text{diabetic}, P < 0.05$; transplant $> \text{diabetic} P < 0.05$), consistent with a normalization of glucose tolerance in the transplant group.

Transplantation of pig pancreatic anlagen into rats. To establish the feasibility of pig-to-rodent xenotransplantation of pancreatic anlagen, we implanted five pancreatic anlagen from E29 pig embryos into each of five adult Lewis rats. Although the usefulness of addition of VEGF and HGF in the DMEM-HF12 in which anlagen are stored before placement into hosts...
has not been established for pig pancreatic anlagen, these experiments were carried out using the growth factors. As for data shown in Figs. 1 and 2, this group is designated transplant. Five other diabetic rats underwent sham surgery. This group is designated diabetic. Rats in the transplant and diabetic groups had levels of glucose measured during the next 20 wk in parallel with a group of five nondiabetic rats (control).

As was the case for experiments in which rat anlagen were transplanted into rats (Fig. 1), levels of glucose in the transplant group that were elevated significantly \((P < 0.05)\) relative to the control group at 1 wk postimplantation were no longer elevated (were normalized) at 2 wk posttransplantation or at later times (3–20 wk) in rats that received pig pancreas implants (Fig. 4). In contrast, the levels of glucose in the diabetic group were elevated significantly relative to controls at all times at which measurements were made.

Before induction of diabetes in diabetic and transplant groups (week 0), weights were slightly higher \((P < 0.05)\) for the control group relative to the diabetic and transplant groups. At weeks 3, 6, and 10 postinduction of diabetes, weights remained higher in the control group relative to the diabetic group. However, weights in control and transplant groups were no longer significantly different (Fig. 5). Therefore, normalization of glucose levels in the transplant group occurred in the context of a normalization of rat weight.

A second set of pig-to-rat transplantations was performed to characterize the response of transplanted rats to a glucose challenge. At 6 wk of posttransplantation, \(k\) values for glucose disappearance in control, diabetic, and transplant groups were 1.61 ± 0.08, 0.29 ± 0.01, and 2.51 ± 0.46 %/min, respectively \((n = 4\) rats per group; control > diabetic, \(P < 0.01\); transplant > diabetic \(P < 0.01\)), consistent with a normalization of glucose tolerance in the transplant group.

We next performed experiments to characterize the histology of transplanted pig pancreatic anlagen. Figure 6A illustrates a section of a duodenum obtained from an E29 domestic pig embryo. The dorsal pancreas and ventral pancreas are labeled. An anti-insulin antibody-stained section of another E29 pig pancreas shows ducts that contain some insulin-positive (red) cells (arrows) surrounded by undifferentiated cells (Fig. 6B). Figure 6C depicts an H&E-stained section of rat omentum obtained 3 wk posttransplantation from five pig pancreatic anlagen. Scattered bits of tissue are found amidst omental fat. Figure 6, D and E, shows, at higher power, control antibody and anti-insulin-stained sections of omentum, respectively. Positive (red) staining for insulin is observed in Fig. 6E. An anti-insulin-stained section photographed at still higher power shows adipocytes surrounding clusters of insulin-staining cells (Fig. 6F). Unlike the case after transplantation of rat pancreas anlagen (21) (Fig. 3), no defined islets can be identified at this time in the implanted embryonic pig pancreas tissue.

To confirm that the insulin-staining tissue shown in the rat omentum illustrated in Fig. 6 is porcine in origin, and to determine whether the porcine pancreatic tissue may have engrafted at locations in addition to the rat omentum, we performed RT-PCR. We first showed that our probes for rat and pig insulin RNA are specific (Fig. 7A). As would be expected for specific probes, no pig insulin mRNA can be detected in total RNA extracted from rat pancreas (R-p) (lane 1). In contrast, the pig primers amplify a band of 243 bp in RNA originating from pig pancreas (P-p), corresponding to pig insulin mRNA (lane 2). The PCR product shown in lane 2 can be cut into three bands of expected sizes (141, 83, and 9 bp, the latter too faint to be reproduced) by BglII (lane 3). The rat primers amplify a band of 241 bp corresponding to rat insulin 2 mRNA in rat pancreas RNA (lane 4). This PCR product can be cut into two bands of expected sizes (189 and 52 bp) by BamHI (lane 5). No amplification occurs in pig pancreas if rat primers are substituted for pig primers (lane 6). Consistent with the absence of BglII restriction sites in rat insulin RNA and the absence of BamHI restriction sites in pig insulin RNA, the rat PCR product is not cut by BglII, and the pig PCR product is not cut by BamHI (data not shown).

Figure 7B shows that the pig primers amplify the band of 243 bp, corresponding to pig insulin (lane 6) with the corresponding BglII digestion products (lane 7) in pig pancreas, and also in rat omentum into which five pig pancreatic anlagen had been transplanted 3 wk previously (lane 3). In contrast, no pig insulin mRNA is detectable in kidney (lane 1), liver (lane 2), pancreas (lane 4), or spleen (lane 5) from the transplanted rats or in RNA isolated from a normal rat pancreas (lane 8). The bands in lanes 1 and 5 are nonspecific products. The data shown in Fig. 7B provide strong evidence that, whereas porcine

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**Figure 7.** RT-PCR of RNA extracted from a pancreas from a nontransplanted rat or pig (A) or a pancreas from a nontransplanted pig or rat or from kidney (k), liver (l), spleen (s), or omentum (o) from a rat into which 5 pig pancreatic anlagen had been transplanted 20 wk previously (B). A: lanes 1–6: 0.5 μg of total RNA from rat pancreas (R-p, lanes 1, 4, and 5) or pig pancreas (P-p, lanes 2, 3, and 6) amplified using pig insulin primers (lanes 1–5) or rat insulin primers (lanes 4–6). Lanes 3 and 5: *BglII or BamHI digestion, respectively. B: lanes 1–5: 2.5 μg total RNA from rat (R) amplified with pig insulin primers; lanes 6–7: 0.5 μg total RNA from pig (P) amplified using pig insulin primers. +, pig insulin RNA (lanes 3 and 6). *BglII digestion products (lane 7). Lane 8: 0.5 μg total RNA from rat pancreas amplified using pig insulin primers.
tissue (containing RNA coding for porcine insulin) is present in omentum of the transplanted rats, it is not present in kidney, liver, spleen, or pancreas.

Figure 8 shows tissues from rats into which pancreatic anlagen had been transplanted 20 wk previously. Relative to an age-matched sham-transplanted diabetic control (Fig. 8A), the omentum in transplanted rats contains an increased quantity of fat (Fig. 8B), consistent with the findings of others who transplanted pancreatic anlagen to nonomental sites (1, 4, 7, 9). Omentum from a rat into which a pancreatic anlage from an E12.5 Lewis rat embryo had been implanted 20 wk previously (Fig. 8C) shows characteristic flattened nuclei (8) as does omentum from a Lewis rat into which a pancreatic anlage from an E29 pig embryo had been implanted 20 wk previously (Fig. 8D, arrows). However, omentum from the rat into which the embryonic pig pancreas had been transplanted is more cellular than that into which the rat anlagen had been transplanted, and it also contains cells in clusters with larger rounded nuclei (Fig. 8D, arrows). These cells have the morphology of engrafted transplanted β-cells (i.e., round with large nucleus and granular cytoplasm) (14), do not stain with control antibody (arrow Fig. 8E), are insulin positive (arrow Fig. 8F), and contain homori-positive granules (arrow Fig. 8G) characteristic of β-cells (24).

To confirm that porcine insulin is present in the omentum from Lewis rats into which a pig pancreatic anlage was transplanted 20 wk previously, insulin was measured with a porcine insulin-specific assay in supernatants derived from two samples from an omentum. Levels of porcine insulin were 2.3 ± 1.0 μU/ml.

Shown in Fig. 9, A and B, are control antibody (Fig. 9A) and anti-glucagon (Fig. 9B) stains of whole pancreas from Lewis rats aged 10 wk. Characteristic of rat islets (27), the most intense staining for glucagon is at the periphery of islets (Fig. 9B). Control antibody stains are negative (Fig. 9A). In VEGF- and HGF-treated Lewis rat pancreatic anlagen that had been transplanted into adult Lewis rats 3 wk previously, the most intense staining for glucagons is also at the periphery (Fig. 9D). No staining is observed using control antibody (Fig. 9C). Isolated glucagon-positive cells (Fig. 9F) are present in the omentum of rats that have been transplanted with pig pancreatic anlagen 20 wk previously (Fig. 9F). Control antibody staining of the rat omentum is negative (Fig. 9E).

DISCUSSION

A new therapy for type 1 diabetes mellitus has long been sought though a variety of biological approaches (3, 26). Those applied in humans include whole pancreas and islet allotransplantation. Given existing technology, a major limitation to the use of either modality is the insufficient supply of human organs. Compounding this limitation for islet transplantation is the need to transplant large quantities of islets to achieve insulin sufficiency even for a limited time (3). It has been suggested that the problems inherent in the isolation of islets from donors maintained on life support result in diminished

Fig. 8. Photographs (A, B) and photomicrographs (C-G) of omentum from diabetic rats into which sham transplantation was performed 20 wk previously (A), 5 pig pancreatic anlagen had been transplanted 20 wk previously (B, D-G), or into which 10 rat pancreatic anlagen had been transplanted 20 wk previously (C). A: omentum from a rat that underwent sham transplantation. B: omentum from a rat into which 5 pig pancreatic anlagen had been transplanted 20 wk previously; arrows delineate fat. C: H&E-stained section of omentum from a Lewis rat into which 10 Lewis rat pancreatic anlagen had been transplanted 20 wk previously. D: H&E-stained section of omentum from a rat into which 5 pig pancreatic anlagen had been transplanted 20 wk previously. Arrowheads delineate flattened nuclei; arrows show rounded nuclei. E-G: sections of omentum from a Lewis rat into which 5 pig pancreatic anlagen had been transplanted 20 wk previously; control antibody-stained section (E); anti-insulin antibody-stained section (F); combined Gomori-stained section (G). Arrows delineate cells. Data are representative of 3 experiments. Magnification is shown in B (for A and B), in D (for C and D), and in G (for E-G).
viability (3). Coupled with the limited ability of β-cells within mature islets to replicate, the diminished viability results in a declining pool of functioning engrafted islets over time (3, 26). Theoretically, the insufficient supply of human tissue could be overcome by the use of islets derived from animals, such as pigs (11–12, 18, 20, 23), as an alternative to human islets. However, whether derived from humans or pigs, β-cell mass within transplanted islets does not undergo significant expansion (3, 26). A way to overcome the limited potential for growth or division of islet cells posttransplantation is to transplant developing fetal or neonatal pancreatic tissue (1, 4–7, 9, 14, 16, 21, 25).

The pancreas is derived from two separate primordia (the dorsal and ventral pancreas) (17). In the rat (21 days of gestation), the primordia arise from the duodenum during embryonic day 11 (E11) of development (dorsal pancreas) and from the endoderm of the hepatic diverticulum on day E12.1 (ventral pancreas) and later fuse (E13). The head of the pancreas develops from the ventral anlage and the tail from the dorsal anlage. Glandular tissue of the pancreas is formed by the budding and rebudding of cords of cells derived from this primordial mass. The terminal parts gradually take on the characteristics of pancreatic acini, while the more proximal portion forms the ducts from which islets are derived beginning on ~E15 during embryonic development (17).

In pigs (~120 days of gestation), the dorsal and ventral anlagen are formed between E20 and E29. The pig pancreas develops much more slowly than the rat pancreas. Cells moderately immunoreactive for insulin can be detected at ~4 wk of gestation and, beginning at 13 wk of gestation, cells that are intensely immunoreactive for insulin are distributed throughout the parenchyma. However, it is not until ~10–13 days after birth that the cells cluster together in small islets (2). The slower development of pig pancreas relative to rat during embryogenesis may be reflected by the delay in appearance of mature β-cells posttransplantation of pig pancreatic anlagen into rat (Fig. 6) relative to rat pancreatic anlagen (Fig. 3), which could explain the finding that, unlike the case for rat transplants (Fig. 3), a stromal component is not a part of the mature graft (Figs. 8 and 9). Whatever the case may be, herein is delineated a novel means by which transplantation of what in effect become individual functioning β-cells (Fig. 8) and α-cells (Fig. 9) can be performed across a highly disparate xenogeneic barrier.

It is somewhat surprising that E29 pig pancreatic anlagen can be transplanted successfully into nonimmunosuppressed Lewis rats. Our ability to do so cannot be explained by the use of anlagen from E29 pig embryos per se, because E29 renal anlagen do not engraft unless Lewis rat hosts are treated with costimulatory blocking agents (13). It cannot be explained as characteristic for embryonic pancreas transplanted into Lewis rats per se, because pancreatic anlagen from E17 buffalo or F344 rat embryos are rejected posttransplantation into adult Lewis hosts (25). It is unlikely to be explicable on the basis of using pancreatic anlagen obtained early during organogenesis,
because pancreatic anlagen obtained at a comparable stage from E12.5 Lewis rat embryos do not engraft in C57Bl/6J mice unless the hosts are treated with costimulatory blocking agents (21). It is not characteristic of porcine pancreatic tissue per se, because adult islets undergo rejection within a week posttransplantation beneath the renal capsule of Lewis rats (28).

An advantage for transplantation of nonvascularized porcine fetal pancreatic tissue relative to developed pancreas is that the former is less subject to hyperacute rejection after transplantation across a discordant xenogeneic barrier (pig to human) (12, 18, 20). However, porcine fetal pancreatic clusters are subject to acute rejection when transplanted into nonimmunosuppressed rodents (18, 20) or susceptible primates such as humans (12).

Castaing et al. (7) transplanted pancreatic anlagen obtained from 6- to 9-wk-old human embryos beneath the renal capsule of β-cell-deficient immunodeficient (NOD/SCID) mice. These investigators found that the transplantation of one embryonic pancreatic tissue resulted, within 6 mo, in control of alloxan-induced hyperglycemia in the mice. The human pancreatic tissue grew, increasing in weight 200 times, and endocrine cells differentiated, the number of human β-cells expanding by a factor of 5,000 (7). After differentiation of transplanted tissue, islets of Langerhans were found amidst stromal tissue (7), as in our rat-to-rat transplants (Fig. 3).

The differences in morphology of developed endocrine tissue between our pig-to-rat transplants (Figs. 7 and 8) and the human-to-mouse transplants of Castaing et al. (7) may be explained by a component of rejection posttransplantation of pig pancreatic anlagen into immune-competent Lewis rats. Indeed, our studies do not establish definitively that E29 porcine pancreatic anlagen do not reject posttransplantation into Lewis rats. However, our data show that immunosuppression is not required for engraftment of E29 pig pancreatic anlagen such that glucose tolerance can be normalized long term (20 wk). Delineation of the host immune response permissive of such engraftment is important but beyond the scope of the present studies.

Immunosuppression of humans after whole pancreas or islet transplantation introduces a set of comorbidities resulting from the use of immunosuppressive agents per se (26). In this context, our finding that pig pancreatic anlagen transplantation can normalize glucose tolerance and restore the nondiabetic pattern of weight gain in nonimmunosuppressed diabetic rats is important, because it establishes a priority for further examination of their utility as xenotransplants to increase functioning β-cell mass in human type 1 diabetics, possibly in the absence of immunosuppression.

GRANTS

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