Growth and Functional Maturation of β-Cells in Implants of Endocrine Cells Purified From Prenatal Porcine Pancreas

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The development of islet cell transplantation as a cure for diabetes is limited by the shortage of human donor organs. Moreover, currently used grafts exhibit a marginal β-cell mass with an apparently low capacity for β-cell renewal and growth. Although duct-associated nonendocrine cells have often been suggested as a potential source for β-cell production, recent work in mice has demonstrated the role of β-cells in postnatal growth of the pancreatic β-cell mass. The present study investigated whether the β-cell mass can grow in implants that are virtually devoid of nonendocrine cells. Endocrine islet cells were purified from prenatal porcine pancreases (gestation >110 days) and implanted under the kidney capsule of nude mice. β-Cells initially presented with signs of immaturity: small size, low insulin content, undetectable C-peptide release, and an inability to correct hyperglycemia. They exhibited a proliferative activity that was highest during posttransplant week 1 (2.6 and 5% bromodeoxyuridine [BrdU]-positive β-cells 4 and 72 h posttransplant) and then decreased over 20 weeks to rates measured in the pancreas (0.2% BrdU-positive cells). β-Cell proliferation in implants first compensated for β-cell loss during posttransplant week 1 and then increased the β-cell number fourfold between posttransplant weeks 1 and 20. Rates of α-cell proliferation were only shortly and moderately increased, which explained the shift in cellular composition of the implant (β-cell 40 vs. 90% and α-cell 40 vs. 7% at the start and posttransplant week 20, respectively). β-Cells progressively matured during the 20 weeks after transplantation, with a twofold increase in cell volume, a sixfold increase in cellular insulin content, plasma C-peptide levels of 1–2 ng/ml, and an ability to correct diabetes. They became structurally organized as homogenous clusters with their secretory vesicles polarized toward fenestrated capillaries. We concluded that the immature β-cell phenotype provides grafts with a marked potential for β-cell growth and differentiation and hence may have a potential role in curing diabetes. Cells with this phenotype can be isolated from prenatal organs; their presence in postnatal organs needs to be investigated. Diabetes 54:3387–3394, 2005

RESEARCH DESIGN AND METHODS

Prenatal porcine pancreatic endocrine cells were obtained from Beta Cell (Zellik, Belgium). They were prepared according to a procedure developed in our laboratory using fetuses of Belgian land race sows after 110–115 days of gestation. Pancreases were dissociated by enzymatic digestion with collagenase P (Roche, Indianapolis, IN; 0.5 mg/ml) and trituration in calcium-free medium. Single endocrine cells were isolated by counterflow elutriation, purified by gradient centrifugation, and cultured in serum-free HAM’s F10 medium containing 6 mmol/l glucose, 5 mmol/l nicotinamide, 2 mmol/l...
glutamine, 50 μmol/l isobutylmethylxanthine, 10⁻³ mol/l hydrocortisone, and 0.5% BSA. At the start and after 1 week of culture, samples were taken to determine their cellular composition and hormone content (25,24). Grafts composed of >90% pure endocrine islet cells and containing 0.5-1 million β-cells were prepared from human organ donor pancreata for 1 week.

**Transplantation of porcine pancreatic endocrine cells in nude mice.** Male immunodeficient nu/nu BALB/c ByJCo mice (age 8 weeks; Iffa Credo, France) were selected as recipients. Animals were maintained in acclimatized rooms with free access to water and pellet food. Transplants were carried out in nondiabetic and diabetic animals. Diabetes was induced by intravenous injection of alloxan (90 mg/kg; Sigma, Munich, Germany) the day before transplantation. Only animals with nonfasting glycemia >500 mg/dl were included in the study because most of them died. During the next 2 weeks irrespective of the presence of a fetal β-cell implant, a “life-saving” rat islet graft was placed under the contralateral kidney capsule until the time that the fetal islet implants had grown and matured according to the studies in nondiabetic recipients; the islet-bearing kidney was then removed and the animals were followed metabolically. In the study of Korbutt et al. (15), such a “life-saving” rat islet graft was not necessary, possibly because a more differentiated neonatal pig islet cell preparation was used following pretreatment with high nicotinamide (10 μmol/l), which has been reported to favor β-cell differentiation (25). Another reason could have been a lower degree of β-cell depletion after alloxan in the Korbutt et al. study. The study protocols were approved by the Ethical Committee of Brussels Free University-VUB and carried out in accordance with Belgian and European Union regulations. Mice were immunosuppressed with 2,2,2 trimethoxyethanol solution (Avertin; Sigma; 500 mg/kg body wt i.p.).

**Analysis of cellular composition of grafts and implants.** Tissue samples were fixed in formalin and paraffin sections were incubated overnight with rabbit anti-synaptophysin (1:20; Dako, Glostrup, Denmark), guinea pig anti-insulin (1:5,000) (27), rabbit anti-glucagon (1:5,000) (27), rabbit anti-somatostatin (1:5,000) (27), or rabbit anti-paracrine polypeptide (1:50,000); gift from Dr. R.E. Chance, Lilly, Indianapolis, IN) (26). After the sections were washed (pH 7.4), they were incubated with peroxidase-labeled anti-mouse (1:20; Amersham, Amersham, U.K.), anti-rabbit (1:20; Amersham), or anti–guinea pig (1:100; Cappel, Aurora, OH) secondary antibody for 30 min at room temperature. The peroxidase reaction was developed by incubating sections in 0.3% H2O2 and 0.15% 3,3’-diaminobenzenedine tetrachloride (Sigma, St. Louis, MO). For the double-labeling immunohistochemistry, anti-synaptophysin Ig was labeled with fluorescein isothiocyanate and Cy3-labeled anti-mouse Ig secondary antibodies (Jackson Immunoresearch, West Grove, PA) were used. Proliferating cells were detected with a BrdU antibody (1:20; Cappel) and identified by an antibody against synaptophysin (28), insulin, glucagon, somatostatin, or pancreatic polypeptide. For each population positive for the endocrine marker, the percentage of BrdU-positive cells was determined and expressed as the proliferation index. For each implant, at least 2,000 cells were analyzed; no regional differences in structural organization were noticed. We also counted the percentage of insulin-positive cells with signs of apoptosis after a 1-h incubation with BrdU.

**RESULTS**

**Characteristics of fetal porcine β-cell graft.** Fetal porcine islet cell grafts were composed of >90% endocrine cells, which mainly corresponded to insulin- and glucagon-positive cells in similar proportions (each representing 39 ± 5% of all cells; somatostatin-positive (7 ± 2%) and pancreatic polypeptide—positive (6 ± 3%) cells were much less abundant (Fig. 1). The remaining cells (<10%) corresponded to cytokeratin (CK-7)-positive cells (5.1 ± 2.3% of all cells) and damaged cells. We observed that >90% of the cells were positive for synaptophysin, indicating that this neuroendocrine marker can be used to identify fetal pancreatic endocrine cells. The CK-7 positive cells were copurified epithelial ductal cells (32). Grafts contained 0.8 ± 0.1 × 10⁶ β-cells and 4.6 ± 1.6 μg insulin; the insulin content of the β-cells averaged 5.2 μg/10⁶ cells. β-Cells had an average cellular volume of 800 μm³, which is comparable with that of neonatal rat β-cells (33) and smaller than that of adult rodent β-cells (30,33). Electron microscopy illustrated endocrine cell aggregates with intact ultrastructure (<5% dead cells). Few apoptotic β-cells were detected (0.5 ± 0.2%). Under basal serum-free culture conditions, 0.5 ± 0.3% of insulin cells were BrdU positive after a 1-h incubation with BrdU.

**Change in cellular composition of fetal porcine β-cell implants.** At posttransplant day 1, the composition of the implant (Fig. 2) was similar to that of the isolated graft, with equal percentages of insulin- (39 ± 5%) and glucagon- (39 ± 5%) positive cells, each contributing similar total cell volumes to the endocrine volume of the implant. During posttransplant week 1, no change occurred in the volume of the insulin cell population. However, that of glucagon cells decreased by 40% (Table 1) in association with a decrease in the percentage of glucagon cells and an increase in the percentage of insulin cells (Fig. 2). From posttransplant week 1 onward, the total volume of insulin cells increased with time (threefold between posttransplant weeks 1 and 10, twofold between posttransplant weeks 10 and 20) (Table 1); this was not the case for the total volume of glucagon cells, which remained stable (Table 1). The proportion of insulin cells further increased to 89% at posttransplant week 20, whereas that of glucagon cells decreased to 7% (Fig. 2).

The increase in the total volume of insulin cells was caused by an increase in the mean individual cell volume as well as an increase in the number of β-cells. Between posttransplant weeks 1 and 20, the mean individual volume of insulin cells almost doubled (to an average volume of 1,500 μm³) whereas the number of insulin cells increased fourfold (Fig. 3). No significant difference was detected between the number of β-cells on posttransplant day 1 and at posttransplant week 1 (Fig. 3). The increase in the total volume of insulin cells between posttransplant weeks 1 and 20 was accompanied by an increase in insulin content of the implant rising to 17- and 50-fold higher values, respectively, at posttransplant weeks 10 and 20.
(Fig. 3). This increase in insulin content of the implant was much more pronounced than the increase in β-cell number or the total volume of insulin cells, which indicated that the β-cells increased their individual insulin store. At posttransplant week 20, the insulin content of the β-cells averaged 31 µg/10⁶ cells, which was sixfold higher than at the start.

**Structural organization of fetal porcine β-cell implants.** As well as changing their cellular composition, implants underwent a structural organization. At posttransplant day 1, they were composed of dispersed single cells, small cell aggregates, and blood cells without structural organization (Fig. 1). At posttransplant week 20, the implants appeared as a white compact mass on the kidney surface, with a diameter of 3–8 mm and clearly visible interconnected capillaries (Fig. 4 A). When viewed microscopically, they presented a homogenous assembly of endocrine cell clusters (Fig. 4 B) that were predominantly formed by insulin-positive cells with a few other endocrine islet cells scattered at their border (Fig. 4 C and D). The space in the center of some endocrine clusters (Fig. 4 C) was filled with erythrocytes without endothelial cell lining (Fig. 4 E). These lacunae do not exhibit the tubular or matrix pattern that is characteristic for vasculogenic mimicry; their formation and functional significance is so far unknown. Within the endocrine cell masses, capillaries with fenestrated endothelial cells were detected, toward which the endocrine cells were oriented with their pole of secretory vesicles (Fig. 4 E and F, arrows).

**Rates of β-cell proliferation and apoptosis in fetal porcine β-cell implants.** During posttransplant week 1, 1–6% of insulin cells were positive for BrdU 1 h after this compound was injected (Fig. 4 G). This proliferation index was already elevated 4 h after transplant (2.6 ± 0.7%) and reached peak values of 5 ± 0.7% after 72 h (Fig. 5). Between posttransplant week 1 and 10, the percentage of BrdU-positive insulin cells remained significantly higher than in the prenatal pancreas in situ (0.2%) but then slowly decreased with time to 0.3% at posttransplant week 20. The percentage of BrdU-positive insulin cells decreased during the first week (2.3% after 72 h) and returned to basal levels from posttransplant week 1 and beyond (0.3% BrdU positive cells).

The first 72 h after the transplant were also characterized by a higher rate of insulin cell apoptosis (1–4%) (Fig. 5).

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**TABLE 1**

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Week 1</th>
<th>Week 10</th>
<th>Week 20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synaptophysin</td>
<td>55 ± 28</td>
<td>45 ± 12</td>
<td>95 ± 29*†</td>
<td>197 ± 60**†</td>
</tr>
<tr>
<td>Insulin</td>
<td>27 ± 13</td>
<td>25 ± 5</td>
<td>77 ± 29*†</td>
<td>180 ± 63**†</td>
</tr>
<tr>
<td>Glucagon</td>
<td>39 ± 11</td>
<td>14 ± 7</td>
<td>11 ± 2</td>
<td>11 ± 6</td>
</tr>
</tbody>
</table>

Data are means ± SD of measurements for three to six mice per time point. Cell volume is given as 10⁷ µm³. *P < 0.05 vs. day 1; †P < 0.05 vs. week 1.
from those in nontransplanted controls during the first 5 posttransplant weeks (91 ± 9 vs. 94 ± 8 mg/dl) but then progressively decreased to reach a level at and beyond posttransplant week 15 of 59 ± 27 mg/dl, which was significantly lower than the level in normal controls (110 ± 10 mg/dl; \( P < 0.05 \)) (Fig. 6). This metabolic effect was associated with a 50% decrease in pancreatic insulin content (25 ± 6 vs. 50 ± 7 \( \mu g \) in sham-operated normal controls; \( P < 0.05 \)).

When fetal porcine islet cell preparations of similar size and composition were implanted in alloxan-induced diabetic nude mice, they did not immediately correct the hyperglycemic state and the mice died during the first 2 weeks after the transplant. This outcome indicated the metabolic incapacity of the fetal porcine \( \beta \)-cell implant during the first posttransplant weeks and correlated with undetectable circulating and low tissue levels of porcine C-peptide or insulin during this period. We therefore examined the metabolic capacity of porcine \( \beta \)-cell implants at a later time when they had undergone the above-described changes in composition and organization and had reached plateau levels of circulating C-peptide (i.e., beyond posttransplant week 10). To this end, the fetal pig \( \beta \)-cell preparations were transplanted in diabetic mice that simultaneously received isolated adult rat islets under the contralateral kidney capsule. The rat islet implant established normoglycemia (Fig. 7) and thus prevented the death of these animals. The rise in circulating porcine C-peptide levels was similar to that in non–alloxan-administered recipients that had received a fetal pig \( \beta \)-cell graft (Fig. 7). Removal of the rat islet implant at posttransplant week 15 did not alter porcine C-peptide levels nor did it reverse the normoglycemic state, indicating that the fetal porcine \( \beta \)-cell implant contained a metabolically responsive and adequate \( \beta \)-cell mass. When the animals were killed at posttransplant week 17, their pancreatic insulin content was in the diabetic range (1.9 ± 1.2 \( \mu g \) [\( n = 6 \)] vs. 34 ± 11 \( \mu g \) in non–alloxan-administered recipients of fetal porcine \( \beta \)-cell grafts) whereas the insulin content of their implant (60 ± 29 \( \mu g \)) was similar to that of the non–alloxan-administered recipients (87 ± 29 \( \mu g \) [\( n = 7 \)]; \( P > 0.05 \)).

DISCUSSION

The shortage in human donor organs has raised interests in pigs as a potential source of islet cell grafts for diabetic patients. Techniques have been developed for preparing insulin-producing tissue from fetal, neonatal, and adult pig pancreases (14,15,20,34). Grafts from all donor ages have been shown to normalize diabetes in immunodeficient patients; instead, a progressive decline in \( \beta \)-cell mass has been observed with time and is considered to be responsible for the loss of metabolic control in long-term implants (9,10,42). On the other hand, implants of fetal pancreatic tissue grow in total volume, \( \beta \)-cell mass, and

4H), which was markedly higher than in the graft before transplantation (0.5 ± 0.2%). This index decreased to <0.2% at posttransplant week 1 or later (Fig. 5).

**Metabolic effects of fetal porcine \( \beta \)-cell implants.** Porcine C-peptide was not detectable in plasma during the first 2 posttransplant weeks but was consistently measured from posttransplant week 5 and beyond, increasing to plateau levels of 1.0–1.5 ng/ml at posttransplant weeks 10–20 (Fig. 6). Blood glucose levels were not different

![Graph of \( \beta \)-cell mass and insulin content](image-url)
insulin content, bringing their overall function with time to a state that corrects hyperglycemia (15,18,19,22). The rapidity of metabolic normalization may vary with the amount of tissue and its cellular composition and also with the stage of development and the species. For porcine islet cell clusters isolated at gestation days 50–70 and implanted under the kidney capsule of nude mice, the correction of diabetes is achieved after only 16–20 weeks; this period can be shortened by the administration of nicotinamide, which is considered to stimulate β-cell differentiation (25,43). This was also the case for porcine neonatal islet cell clusters (15). The implants undergo a maturation process that seems to involve both neoformation and functional maturation of β-cells (19,22,43). The relative contribution of both components is not yet clear. It is also unknown to which extent new β-cells are formed from nonendocrine cells or other β-cells (22). Most authors underline the importance of a differentiation of nonendocrine cells to β-cells (19,20,22,44). At the time of implantation, these grafts are indeed mainly composed of nonendocrine cells; the progressive disappearance of the nonendocrine cells is found to be compatible with a transition to β-cells. The nonendocrine-to-endocrine differentiation route is also supported by the observation that several β-cells share markers with adjacent nonendocrine cells (22). Moreover, little evidence has been provided for a major contribution of β-cell proliferation. A study comparing the outcome of fetal islet cell clusters with a low percentage of β-cells and fetal β-cell aggregates even concluded that the absence of nonendocrine cells markedly reduced the normalizing capacity of the grafts (44).

To investigate more directly the growth potential of fetal β-cells, we undertook a morphometric study on grafts of purified endocrine islet cells that were virtually devoid of

FIG. 4. Representative images of implants at posttransplantation week 20 (A–F) and of proliferating (G) and apoptotic (H) insulin-positive cells. Macroscopically, the grafts are 3–4 mm in size (A, arrows) and well distinguishable from the kidney parenchyma. Light microscopy shows homogenous endocrine cell clusters (B), in which >90% of the cells stain for insulin (green; C). Glucagon, somatostatin, and PP cells (red) are located at the periphery of these clusters (D). The clusters are surrounded by capillaries (B and E, arrows) with fenestrated endothelial cells (F, arrows). In some endocrine clusters, lacunae were noticed with red blood cells and without endothelial cell lining (E). The endocrine cells display a polarity with the secretory pole oriented towards the capillaries (E). Cells coimmunostained for insulin (green) and BrdU (red) (G) were observed in all grafts. Apoptotic insulin-positive cells (H) were detected by propidium iodide (red) staining of cell nuclei on the basis of condensed or fragmented nuclei (arrow). N, nucleus; RBC, red blood cell. The scale bars represent 1 mm (A and C), 25 μm (B), 50 μm (C), 10 μm (D and G), 2 μm (F), 0.2 μm (F), and 20 μm (H).
nonendocrine cells. Purified endocrine islet cell grafts were prepared from late fetal (110–115 days gestation) pig pancreases. They consisted of 90% endocrine cells, with similar percentages (40%) of insulin- and glucagon-positive cells. This composition markedly differed from that of fetal or neonatal porcine islet cell clusters prepared after 50–70 days of gestation (14) or 1–3 days after birth (15,20), which have 5 or 24% -cells and 1 or 8% -cells, respectively. The -cells in our preparation were immature, as determined by comparing them with adult -cells; their cellular volume was twofold smaller (30,33) and their insulin content (5 g/million -cells) was sixfold lower. They were also unable to correct diabetes in nude mice when transplanted in numbers (20 million -cells/kg body wt) that were 5- to 10-fold higher than those of adult -cells that normalize rodent or human diabetes (4,5,7,45,46). The porcine -cells developed a functionally mature state over 10–20 weeks after transplantation, as evidenced by their progressive increase in size and insulin content, the appearance of circulating porcine C-peptide, and their progressive gain of metabolic control.

In addition to presenting signs of maturing -cells, the endocrine cell implants also exhibited a continuous formation of new -cells. The number of -cells increased 4.3-fold between the end of posttransplant week 1 and posttransplant week 20. This increase could not be attributed to nonendocrine cells as these were virtually absent (<1% at the end of posttransplant week 1). Instead, it was due to the high proliferative activity of the -cells: at posttransplant week 1, the percentage of BrdU-positive -cells was sixfold higher than in the prenatal pancreas in situ and remained elevated, although at a lower level, until posttransplant week 10. This index was also sixfold higher than in the same preparation tested in vitro in the absence of serum. Factors released during the inflammatory and tissue repair process probably stimulated the -cells that are prone to proliferation. Only 1 day after the implantation injury, the proliferating fibroblasts and endothelial cells were noticed and 2 days later, granulation tissue with small blood vessels was observed. Despite this increased rate of -cell proliferation during posttransplant week 1, neither the number nor total volume of -cells increased. This occurrence might have reflected a simultaneous equal loss of -cells that was compatible with the elevated percentages of apoptotic -cells during this period. Be-
cause apoptotic cells are rapidly cleared, it is not possible to calculate the cell loss from the percentages of apoptotic β-cells. Apoptotic α-cells were found in similar percentages, an observation that is consistent with nonspecific damage in the implant site. However, there were less BrdU-positive glucagon cells than insulin cells (2 vs. 5% at day 3), which explains the reduction in total volume of this cell population at posttransplant week 1. The rates of BrdU-positive glucagon cells remained low during the entire study period in contrast to our observations of β-cells. The quantitative differences between both cell populations thus further increased with time, resulting in a posttransplant week 20 in an implant with 80% β-cells and only 7% α-cells. An abundance of β-over α-cells has been previously described for grafts prepared from fetal and neonatal pancreatic tissue as well as for adult islet grafts (15,19,46,47).

The observed growth in β-cell number and individual cell volume did occur under normoglycemic conditions, as maintained by the mouse endocrine pancreas or a rat islet graft. Under this condition, the implant induced, from posttransplant week 10 on, plasma porcine C-peptide levels of 1–2 ng/ml. This level was maintained when the rat islet graft was removed; it corresponded to a β-cell mass that had grown and matured to a higher insulin content than in the normal mouse pancreas. At this level of growth and differentiation, the porcine β-cell implant prevented the development of diabetes, with no decline in its insulin reserves. Morphological examination revealed that the metabolically adequate porcine fetal β-cell implant was structurally organized in contrast to the randomly distributed small cell clusters in the initial implant. It consisted of homogenous β-cell clusters with peripherally scattered α-cells surrounded by fenestrated capillaries, as described in normal islet tissue (48). The β-cells presented intracellular signs of polarity, with their pole of secretory vesicles located at the pole that faced the capillaries. Between the clusters, blood-filled lacunae devoid of an endothelial lining were also noticed. The revascularization process proceeded throughout the 20-week study period, as evidenced by an ongoing capillary sprouting.

The present study showed that endocrine islet cells can be purified from porcine fetal pancreases, opening the potential for large scale isolations of β-cells. Our preparations contained 40% β-cells with signs of immaturity. When transplanted under the kidney capsule of normoglycemic mice, the β-cells exhibited a prolonged proliferating activity, first compensating for β-cell loss during engraftment and then increasing the β-cell number fourfold over the subsequent 20 weeks. These data indicate that immature β-cells can achieve a sustained renewal and growth of the β-cell mass. It is conceivable that immature β-cells were also responsible for the growth of the β-cell mass that has been observed in human duct cell preparations isolated from young human donors (27). Our observations are consistent with recent work showing that the proliferation of β-cells is the main mechanism for growth of the β-cell mass in the postnatal mouse pancreas (49,50). They also illustrate that impure islet cell preparations are not needed to achieve β-cell renewal or adaptive growth in implants of pancreatic tissue.

FIG. 7. Plasma glucose and porcine C-peptide levels in alloxan-induced diabetic nude mice that received a prenatal porcine β-cell implant under the left kidney capsule and an adult rat islet implant under the right kidney capsule (○; n = 6), and non-alloxan-administered control nude mice that received a prenatal porcine β-cell implant under the left kidney capsule and a sham operation in the right kidney (●; n = 7). Results are means ± SD of measurements for the listed number of mice. Nx, nephrectomy of right kidneys; Tx, transplantation. *Significant difference vs. controls (Kruskal-Wallis test, P < 0.05).

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