Small rat islets are superior to large islets in in vitro function and in transplantation outcomes

R. R. MacGregor, S. J. Williams, P. Y. Tong, K. Kover, W. V. Moore, and L. Stehno-Bittel. Small rat islets are superior to large islets in in vitro function and in transplantation outcomes. Am J Physiol Endocrinol Metab 290: E771–E779, 2006. First published November 22, 2005; doi:10.1152/ajpendo.00097.2005.—Barriers to the use of islet transplantation as a practical treatment for diabetes include the limited number of available donor pancreata. This project was designed to determine whether the size of the islet could influence the success rate of islet transplantations in rats. Islets from adult rats were divided into two groups containing small (diameter <125 μm) or large (diameter >150 μm) islets. An average pancreas yielded three times more small islets than large. Smaller islets were ~20% more viable, with large islets containing a scattered pattern of necrotic and apoptotic cells or central core cell death. Small islets in culture consumed twice as much oxygen as large islets when normalized for the same islet equivalents. In static incubation, small islets released three times more insulin under basal conditions than did large islets. During exposure to high glucose conditions, the small islets released four times more insulin than the same islet equivalents of large islets, and five times more insulin was released by the small islets in response to glucose and depolarization with K+. Most importantly, the small islets were far superior to large islets when transplanted into diabetic animals. When marginal islet equivalencies were used for renal subcapsular transplantation, large islets failed to produce euglycemia in any recipient rats, whereas small islets were successful 80% of the time. The results indicate that small islets are superior to large islets in in vitro testing and for transplantation into the kidney capsule of diabetic rats.

islet transplant; insulin secretion; viability

THE RISE IN CASES OF DIABETES MELLITUS in the United States has been called an epidemic. It is the third leading cause of death by disease and rivals heart disease and cancer as a major killer of United States citizens. For unexplained reasons the occurrence of type 1 diabetes is increasing worldwide, and the age of onset has decreased by 3–5 yr over the past decade so that many children now develop diabetes before entering school. The result is that more people with diabetes will spend a larger percentage of their life at risk for developing the chronic complications related to type 1 diabetes. Because the risk for development of most of the chronic complications associated with diabetes is related to glycemic control, significant attention is directed toward novel therapies, such as islet transplantation, to improve glycemic control.

Islet transplants in humans were first attempted in the 1980s (7, 8). Initial success rates for human islet transplantation were disappointing, with only 5% of patients receiving transplants achieving partial function (19). Amid the failures were isolated success stories of individuals achieving prolonged reversal of their diabetes for a 1- to 2-yr period (19), which encouraged researchers to continue this approach for the treatment of diabetes. In 2000, islet transplantations were performed successfully on seven patients with diabetes by using a suppression regimen that omitted glucocorticoids, now referred to as the Edmonton protocol (17). Islet transplantation outcomes have improved markedly, with current success rates up to 80% (18). Regardless of the optimism generated by these results, barriers to the use of islet transplantation as a practical treatment for diabetes still exist, in particular the limited number of available donor pancreata. In addition, most diabetic patients still require multiple transplants to achieve insulin independence. Present techniques for isolation of human islets result in islet preparations that apparently lose at least 50% of the islets over the 10 wk immediately following transplantation (16). Furthermore, there are presently no reliable markers for predicting which population of islets will result in successful transplants.

We have observed that isolated rat islets of large diameter could not be maintained in culture as well as islets with smaller (<100 μm) diameters. In human islet isolation procedures, the small diameter islets are usually ignored because they represent a relatively small fraction of total islet volume. This project was designed to determine whether the size of the islet could influence the success rate of islet transplantations in rats.

MATERIALS AND METHODS

Rat Islet Isolation and Separation Procedures

Adult male dark agouti (DA) rats were anesthetized by intraperitoneal injection of a mixture of ketamine and xylazine. All procedures used in this study were approved by the Institutional Animal Care and Use Committee. The peritoneal cavity was exposed and the pancreatic ductal connection to the intestine clamped. The pancreas was cannulated in situ via the common bile duct and distended by pumping a cold solution of collagenase into the duct. Collagenase (CLS-1; Worthington Biochemical, Lakewood, NJ) was dissolved in 20 ml of Leibovitz L15 at 450 U/ml. Subsequently, the distended pancreas was excised, transferred to 50 ml centrifuge tubes, and incubated for 20–30 min with gentle tumbling in a 37°C incubator. After incubation, the tube was gently shaken to dislodge islets. The contents of the tube were placed in diluted ice-cold Hank’s balanced salt solution (HBSS) containing 10% of newborn calf serum. The digest was allowed to settle at 1 g, and the supernatant was removed. More HBSS serum was added and the process repeated. The washed digest was
passed through a 500-μm stainless steel screen and sedimented for 1 min at 300 g in a refrigerated centrifuge. The pellet was mixed with 10 ml of 1.110 g/ml Histopaque (density = 1.1085; Sigma Diagnostics, St. Louis, MO) and centrifuged for 10 min at 800 g. The islets floating on the gradient were collected and sedimented separately, then placed into Ham’s F-12 culture medium containing 10% of fetal bovine serum and put into a 37°C culture chamber containing 5% CO₂.

**Yield measurements.** Triplicate samples of each batch of islets were examined, each comprising ~2% of the islet fraction. Individual islets were counted and their diameters measured. For irregularly shaped islets, three to four diameter measurements were taken at different locations on the islet and the average was used. Islet volumes were calculated and converted to islet equivalents for the sample and the entire islet fraction. Small islets were defined as those having a diameter of <125 μm, whereas large islets had a diameter of ≥150 μm.

**Separation of small from large islets.** Fresh islets or islets cultured overnight were sedimented and then placed in 1–2 ml of L15 medium. The islets were then quickly layered over a single-step gradient of 5% BSA in L15. Sedimentation at 1 g was permitted to occur for an empirically set period of time until large islets were observed in the bottom of the tube. At that point the top 2 ml (without BSA) of the gradient were discarded, and all but the bottom 2 ml were carefully removed to define the small islet population. The sedimented islets and those in the bottom 2 ml were combined as the large islet fraction. Gradients were repeated if needed to optimize the separation of large and small islets. Final islet fractions were sedimented and placed into culture in a 1:1 mixture of Ham’s F-12 and glucose-free RPMI 1640 (glucose = 5 mM) until glucose sensitivity experiments were performed.

**Fluorescence Confocal Microscopy**

For viability assays, islets were placed in a 500-μl volume of L15 medium with live/dead fluorophores, Sytox (Molecular Probes, 1 μm), and Calcein (Molecular Probes, 0.5 μm) and incubated for 15–30 min at 37°C. When monitoring apoptotic and necrotic cells, islets were incubated in YO-PRO-1 and propidium iodide (Vybrant Apoptosis Assay, Molecular Probes) for 30 min at 37°C. For either assay, islets were rinsed with phosphate buffered saline consisting of (in mM) 137 NaCl, 2.7 KCl, 4.3 Na₂HPO₄, and 1.4 KH₂PO₄, pH 7.4, and placed in the Attofluor Chamber (Molecular Probes) on the Olympus Fluoview 300 confocal microscope housed in the Diabetes Research Laboratory. Images were acquired using ×40 or ×60 objectives. All images were collected within 20 min of removal of the islets from the medium. Three simultaneous images were collected for each islet using He-Ne and argon lasers and a third bright-field image.

Live/dead analysis was completed by identifying the islets in the field and encircling the individual islets using the Fluoview software. Background fluorescence was subtracted from all images. Viability percentages were calculated by developing hue histograms, using Photoshop (Adobe), from the fields of interest and calculating the total pixels in the green (live) and red (dead) hues. The ratio representing the live cells divided by the total islet area was calculated as the percent live value. Islet diameters and perimeters were calculated using Scion software so that viability values could be categorized according to the size of the islet.

When β-cell size was determined, the islets were incubated with dithizone for 15 min, as previously described (13). Individual cell perimeters and diameters in two locations were measured by using the Fluoview software package.

**Oxygen Consumption**

Islet samples, separated by size, and with known islet equivalencies, were placed in Ham’s medium and aliquotted into oxygen-sensing, 96-well microplates (BD Biosciences), employing standard published methods (24). Fluorescence of the individual wells, indicating relative changes in O₂ consumption, was measured on a UV microplate reader (excitation wavelength of 485 nm, emission at 590–630 nm). The values were normalized by subtracting the average of three blank wells from each measured well. Finally, values were divided by islet equivalencies to yield the O₂ consumption/islet equivalency.

**Insulin Secretion (Static and Perifusion)**

**Static incubations.** Small or large islets of equal islet equivalency values (usually 30 IE) were distributed in 24-well plates in RPMI 1640 medium containing 3 mM glucose for a 1-h preincubation period at 37°C and 5% CO₂. For experimental incubations, the islets were incubated for 2 h in fresh medium containing either 3 or 30 mM glucose, or 30 mM glucose containing 25 mM KCl (corrected for osmolality). Triplicate wells for each condition were tested. Medium samples were assayed for insulin content using an ELISA immunoassay (Mercodia; ALPCO). Results were expressed as averages of the triplicate samples ± SE and were compared by Student’s t-test.

**Perifusion incubations.** Equal numbers of islet equivalents (≤300 IE per chamber) of small and large islet groups were placed in perifusion chambers. The medium (RPMI 1640), equilibrated with 5% CO₂, was pumped through the chambers at 1 ml/min. The initial glucose concentration was 3 mM for 1 h followed by exposure to...
medium containing 30 mM glucose for 30 min and then back to 3 mM for 30 min. Finally, medium feeds were switched to medium containing 30 mM glucose and 25 mM K⁺, as described above. Fractions were collected every 10 min, i.e., three samples per condition, and later analyzed for insulin content as described above.

Immunohistochemistry

Islets were fixed in 4% neutral buffered formalin in a microcentrifuge tube at 4° overnight. Low-temperature gelling (4%) agarose (Sigma) was dissolved in water. After the islets had settled to the bottom of the centrifuge tube the neutral buffered formalin was removed, and the islets were resuspended in the 4% agarose solution. The islet-agarose mixture was delivered into a piece of plastic tubing and allowed to gel. The cylindrical-shaped agarose was removed from the tubing by pushing air through the tube with a syringe. The cylindrical sharpened agarose was placed on a small piece of lens paper, and a drop of eosin was added to identify the islets within the agarose. The paper was placed in a cassette for processing to paraffin wax. 6-micrometer sections were cut onto glass and stained with hematoxylin and eosin to illustrate the general morphology of the islets. Antibody staining for detection of insulin and glucagon protein was performed as directed by the manufacturer (Zymed, San Francisco, CA).

Transplantation

Diabetes was induced in the recipient animals by injecting streptozotocin (65 mg/kg) intraperitonally (1 injection). When blood glucose levels were >250 mg/dl for 3 consecutive days, the rats were considered diabetic. Rats were anesthetized with 45 mg/kg pentobarbital sodium. After the rat was shaved and cleaned with betadine scrub, an incision was made in the body wall on the left flank. The kidney was delivered into the wound, and a small incision was made in the kidney capsule. The islets were placed under the capsule using a small bore pipette. The kidney was placed back into its original position and the incision closed with wound clips. Bovine/porcine zinc-insulin (NPH Iletin I) injections (2 times/day) were given to recipients for 3 days postislet transplant to reduce the stress of hyperglycemia on the newly transplanted islets.

Statistics

Unless otherwise noted, all results were tested for significance using hierarchal ANOVA, with P < 0.05. Where appropriate, figures illustrate means ± SE.

RESULTS

Yield

By dividing the small from the large islets, several unique characteristics were noted. Not surprisingly, the numerical yield of small islets from each single pancreas was 3 (± 0.4 SE)-fold greater than the yield of large islets. Detailed accounts of the size and yield from each rat pancreas were obtained. Fig. 1A illustrates a typical yield histogram from a rat islet preparation. The large islets account for 73% of the total islet equivalencies, and the small islets make up the remaining 27% (n = 43 rats), which is consistent with previous publications (23). Note the bimodal distribution of the islets, with a peak at 75–100 μm for the small islets and another smaller peak at 200 μm.
islets” was 50.3 μm in diameter for the group illustrated in the “small islets” were determined from over 300 large and 125 small islets. The results of six rat preparations, with fluorescence measurements collected from over 300 large and 125 small islets. The mean islet diameter for the group illustrated in the “small islets” was 50.3 ± 5.6 μm, whereas large islets averaged 204 ± 16.9 μm in diameter (Fig. 2B). There is clearly a statistical difference between the small and large islet viability with small islets comprised of nearly 98% live cells.

Further investigation into the causes of cell death indicates that both apoptosis and necrosis were responsible for the loss of cells in the large islets. Figure 3 shows another example of core cell death in a large islet maintained in culture for 24 h, this time staining with the apoptosis/necrosis stains. The fluorescence images were overlaid onto the transmitted-light image (gray). Cells lacking fluorescence were alive, whereas red/yellow indicated necrosis and green stained apoptotic cells. The cartoon at the top of the images shows the three planes of image collection. Figure 3A shows that at the base of the islet (near the periphery) there were no necrotic or apoptotic cells. In contrast, at the center of the islet (Fig. 3B) there was significant central core death. Higher into the islet (Fig. 3C) the optical slice showed a smaller area of core death, as the image nicked the upper section. Although an exact spherical shape of core death (as shown in Fig. 3) was unusual, core death itself was noted frequently in the large islets but rarely seen (<4%) in the small islet population. Figure 4A shows a typical small islet stained for apoptosis/necrosis. Only a few β-cells on the periphery of the islet were dead, and those included a combination of necrotic or apoptotic cells. In those large islets that did not have core cell death, a scattered pattern of necrotic and apoptotic cells was noted. Fig. 4B shows a patchwork pattern of necrotic and apoptotic cells in the large islet, which was in agreement with previous reports (6).

Oxygen Consumption

The rate of O2 consumption is indicative of mitochondrial function (9). In pancreatic β-cells it is also correlated with glucose-stimulated insulin secretion (20). We measured O2 consumption using biosensor microplates. Islets were separated into large and small sizes, and islet equivalencies were determined. Equal islet equivalencies were transferred to the O2-sensing microplates. O2 measurements were collected on each well every 24–48 h for the subsequent 10 days. The rate of O2 consumption by the small rat islets was two times higher than the large islet population when normalized for islet equivalencies from days 2 to 6 after isolation (Fig. 5). By day 6, the rate of O2 consumption had reached a plateau that still showed significantly higher O2 consumption by the small islets compared with the large ones. This experiment was repeated eight times, and the results were consistent with the smaller islets consuming a greater level of O2 over the total course of the experiment (P < 0.01).

Insulin Secretion

Static insulin secretion experiments were completed for equal islet equivalencies of large and small rat islets by using insulin-
sensing ELISA. Both small and large islets responded appropriately to the incubation conditions (Fig. 6). Stimulation with high glucose resulted in a robust release of insulin that was five times higher than basal levels. Exposure to high glucose and K+ further increased insulin release to 7.5 times above resting levels. Large islets also released more insulin with stimulation but not to the levels secreted by the small islets. The basal insulin secretion in low glucose levels was three times less than the basal level of release by the small islets (P < 0.01). This trend continued for the large islet release of insulin in all conditions. A simple method for comparing insulin secretion per islet equivalency is to evaluate the ratio of small islet insulin release to large islet release. Table 1, left, illustrates the small- to large-islet ratio of insulin secretion from 14 static incubation experiments, indicating that for every condition the small islets released more insulin than the large ones (ratio > 1).

Perifusion experiments examining the effects of high glucose and high glucose plus K+ on insulin secretion showed similar responses compared with the static incubation experiments for group data. Perfusion insulin secretion results were run with duplicate chambers of large and small islets. Islets were first perfused with low glucose, resulting in similar amounts of insulin secretion from both groups. In response to a challenge with high glucose, both groups of islets increased the amount of insulin released, with the small islets releasing slightly more. Insulin levels returned to basal amounts with a return to the low-glucose environment. Finally, stimulation with high glucose and K+ caused a rapid increase in insulin release from the small islets that was greater than the insulin released from large islets. Table 1, right, illustrates the comparison of secretion between large and small islets averaged from eight perifusion experiments. On average, small islets released 1.7 times more insulin from the high glucose challenge when islets were stimulated with high glucose and K+, they released more than twice the amount of insulin than their large counterparts (P < 0.05).

Fig. 4. A: small islets showed little cell death from either necrosis or apoptosis. As this image illustrates, most dead cells were found on the periphery of the small islets. B: in contrast, large islets had scattered necrotic and apoptotic cells. Note the difference in the scale bars for the 2 images.

Fig. 5. Oxygen consumption was measured in small and large islets using oxygen-sensing microplates. Graph illustrates 1 example in which each measurement was taken in triplicate. OD measurements were taken 1 day after plating and on days 2, 6, 8, and 12. Small islets consumed more oxygen at each time point than large islets.

Fig. 6. Insulin secretion was measured using static incubation. Islets were first exposed to low glucose and then high glucose or high glucose with depolarization (high K+). Large islets demonstrated less insulin release for each condition (*P < 0.01, n = 14 experiments).
Islet Composition

Immunohistochemistry was performed on sectioned islets to determine the distribution of the α- and β-cells (Fig. 7, A and B, respectively) in small and large islets. Both large and small islets showed the typical morphology and distribution of glucagon- and insulin-positive cells, with the α-cells (glucagon positive) located mostly on the periphery of the islet.

We were also interested in any obvious differences in β-cell morphology, as it has been shown that larger β-cells secrete more insulin (5). We measured the cross-sectional diameter of β-cells stained with dithizone from large and small islets. The mean diameter of β-cells was $10.5 \pm 0.2$ mm ($n = 192$ cells) from the small islets and $11.7 \pm 0.1$ mm ($n = 232$ cells) from the large islets, resulting in a statistically significant difference ($P < 0.05$). It is important to note that these measurements were obtained on intact, freshly dispersed islets without fixative, which can alter cell size.

Transplantation

Transplantations of the large or small rat islets were completed ($n = 10$ transplantations/group). Streptozotocin-induced diabetic DA rats received a marginal mass (1,000 IE) of either large (>150 μm) or small (<125 μm) syngeneic islets under the kidney capsule. Blood glucose levels were monitored for 8 wk. Figure 8A shows the results from the first five transplants for each group. All of the recipients of large islets remained hyperglycemic after transplantation (10 of 10). In contrast, 8 of 10 recipients of small islets had blood glucose levels close to or at normal levels 7–10 days after transplantation, which remained normal for the entire 8-wk period. Figure 8B illustrates the mean plasma glucose levels 60 days after transplantation for all 20 animals.

Table 1. Ratio of insulin secretion by small/large pancreatic islets

<table>
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<tr>
<th>Condition</th>
<th>Static Incubation ($n = 14$ experiments)</th>
<th>Perifusion ($n = 8$ experiments)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low glucose</td>
<td>$3.38 \pm 0.73$</td>
<td>$1.73 \pm 0.52$</td>
</tr>
<tr>
<td>High glucose</td>
<td>$4.00 \pm 0.63$</td>
<td>$1.93 \pm 0.26$</td>
</tr>
<tr>
<td>High glucose and K+</td>
<td>$5.12 \pm 0.88$</td>
<td>$2.14 \pm 0.64$</td>
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Values are means ± SE. A comparison of insulin release from small and large islets was calculated with the ratio of small to large islet released insulin. The insulin release was normalized for islet equivalencies by calculating it as ng ml$^{-1}$min$^{-1}$/100 IE. For each condition the small islets released statistically more insulin than the large islets, whether static incubation methods or perifusion conditions were used ($P < 0.05$). Readings for each experiment were taken in duplicate.

Fig. 7. Immunohistochemistry for detection of glucagon and insulin protein was performed on sections of large (left) and small (right) islets. A: red staining in the periphery of large and small islets indicates glucagon-positive cells. B: dispersed red staining throughout the large and small islets indicates insulin-positive cells.
Islet grafts from the kidney capsule were removed 8 wk after transplantation. The tissue was fixed and immunolabeled for insulin. Figure 9 shows the graft from an animal that received small islet transplantation and was euglycemic for the 8 wk. There was substantial staining for insulin in the graft. In contrast, the animal that received the transplantation of large islets continued to be hyperglycemic for the 8-wk period and showed little immunolabeling for insulin in the grafts.

Fig. 9. Islet grafts removed from the kidney capsule 8 wk after transplantation were immunolabeled for insulin. Left: relatively more insulin immunolabeling and an established capillary network in a graft using small islets. In contrast, grafts of large islets showed little insulin immunolabeling and significant fibrosis (right). Images are representative of 4 different animals.
DISCUSSION

Although many factors affecting transplantation success have been studied, one facet of islet function whose investigation has been limited is the effect of the size of islets on their ability to survive and function after transplantation. von Mach et al. (22) showed that large islets did not survive cryopreservation as well as their small counterparts. Likewise, Cui et al. (3) reported that large islets were subject to core cell damage that could be prevented by storage in low temperatures. Hamster islets with diameters <200 μm showed little core damage with 7 days of culture at 37°C. In contrast, islets with diameters >200 μm had core cell death, as measured by terminal deoxyribonucleotidyl-mediated dUTP nick-end labeling assays, and that cell death was greatest in islets >300 μm in diameter. These results are in agreement with the data presented here. Additional reports have published results that support the finding that large islets are prone to decreased viability. For example, small islets from obese rats secreted five times more insulin in vitro than did large islets (2). The authors concluded that at low glucose levels, large islets were most important in insulin secretion, whereas when challenged with high glucose levels, the small islets were essential. This work was done in the type 2 model of diabetic animals, which appears to involve different pathways, because β-cells from type 2 diabetic animals demonstrate hypertrophy (1, 2).

In a report from Tatarkiewicz et al. (21), results implied that small islet subunits were appropriate for transplantation. The small subunits (called pancreatic cell clusters) were able to reverse hyperglycemia after transplantation. The authors were interested in methods to block aggregation of the cells for transplantation (21), and they did not directly test how the pancreatic cell clusters compared with large islets. There have been other reports (4, 11) that indicated differences in the size of the islets. However, those populations of islets were not separated specifically by size but rather by the age of the animal, with younger animals containing smaller islets. In fact, analysis of the size histograms from one of those studies (4) show that up to 79% of the islets from the old pigs (assumed to be large) were <150 μm. The present study is the first attempt to characterize in a detailed manner the effectiveness of small islets for transplantation.

We report here that smaller islets (<125 μm) were superior to larger islets in viability, in vitro functional assays, and transplant outcomes. In fact, an average pancreas yielded three times more small islets than large islets (2). The authors concluded that at low glucose levels, large islets were most important in insulin secretion, whereas when challenged with high glucose levels, the small islets were essential. This work was done in the type 2 model of diabetic animals, which appears to involve different pathways, because β-cells from type 2 diabetic animals demonstrate hypertrophy (1, 2).

A Japanese study (10) reported that small islets far outnumber large islets in human pancreata but constituted only a small percentage of the total islet volume. The report concluded that the bulk of endocrine function in vivo was carried out by the large islets, again based on the assumption that islet volume was the most important predictor of function. Another study (12) has assumed that large islets were superior and thus classified isolation methods on the basis of whether they produced large or small islets. Our data support the argument that small islets may be responsible for a substantial portion of the functional units of the pancreas and may be important to consider for transplantation. Regardless of which islet size carries the greater burden of function in vivo, the situation changes when islets are removed from their vascularized environment. A cell in the center of a 200-μm diameter islet has a 100-μm diffusion barrier to oxygen and nutrients in vitro, whereas an islet of 100 μm diameter has a barrier that is one-half that distance. We propose that after transplantation, small islets may be responsible for the majority of the functional burden in regard to glucose control. Transplanted large islets are the most susceptible to ischemia and starvation caused by the need to survive without a vascular system (15). Mattsson et al. (14) demonstrated that even 1 mo after transplantation, the vascular density of transplanted islets was less than one-half the endogenous vascular supply, irregardless of the site of the transplant. The current view that large islets are better than small ones when preparing the islet preparation for transplantation may need to be modified.

The important findings of this report are that small islets are superior to large islets in in vitro testing and renal subcapsular transplantation. With regard to the mass of transplantable tissue from a single pancreas, the relatively small total mass of the small islets may be compensated by increased functional capacity and viability. We are presently completing the same experiments by using human islets. Additional work must be done to determine the mechanism that would explain the superior function of the small islets. The ability to select superior islets for transplants and a better understanding of the control of function and viability of islets after transplantation will be important in improving the outcomes of islet transplantation.

GRANTS

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REFERENCES


