Evidence of Functional Impairment of Syngeneically Transplanted Mouse Pancreatic Islets Retrieved from the Liver

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A drawback in pancreatic islet transplantation is the large number of islets needed to obtain insulin independence in patients with diabetes. This most likely reflects extensive posttransplantation islet cell death and functional impairment of the remaining endocrine cells. We aimed to develop an experimental method to retrieve transplanted islets from the mouse liver, which would enable comparisons of transplanted and endogenous islets and provide valuable information on functional changes induced by intraportal transplantation. Transplanted islets were obtained by retrograde perfusion of the liver with collagenase. The identity of retrieved tissue as transplanted islets was confirmed by intravital staining, immunohistochemistry, and electron microscopy. The retrieved islets, irrespective of whether they had resided in diabetic or nondiabetic recipients, had a markedly lower insulin content and glucose-stimulated insulin release when compared with isolated endogenous islets. The glucose oxidation rate was also markedly lower in the retrieved islets, suggesting mitochondrial dysfunction. These disturbances in insulin content, insulin release, and glucose oxidation rate were not reversed by a few days of culture after retrieval. The results implicate changes in islet function after intraportal transplantation. Such dysfunction may contribute to the high number of islets needed for successful transplantation in diabetic individuals. Diabetes 53:948–954, 2004

RESEARCH DESIGN AND METHODS

Male C57BL/6 mice that weighed 25–30 g were purchased from Bomholtgaard Research and Breeding Center (Ny, Denmark). The animals had free access to tap water and pelleted food throughout the course of the study. All experiments were approved by the local animal ethics committee for Uppsala University.

Islet isolation and culture. Pancreatic islets were isolated from C57BL/6 mice by a collagenase digestion method, as previously described (12). Some of the isolated islets were allocated directly to measurements of glucose- or theophylline-stimulated insulin release and rate of glucose oxidation, whereas others, in groups of 150 islets, were cultured free-floating for 3–4 days in 5 ml of culture medium consisting of RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with L-glutamine (Sigma-Aldrich), benzylpenicillin (100 units/ml; Roche Diagnostics Scandinavia, Bromma, Sweden), streptomycin (0.1 mg/ml; Sigma-Aldrich), and 10% (vol/vol) FCS (Sigma-Aldrich). Culture medium was changed every second day.

Intraportal transplantation. Groups of 200–250 cultured islets were packed in a butterfly needle (25 gauge) and implanted in a volume of 100–150 μl via the portal vein of syngeneic Avertin-anesthetized C57BL/6 mice (0.02 ml/g body wt intraperitoneally of a 2.5% [vol/vol] solution of 10 g 97% [vol/vol] 2,2,2-trifluoroethanol [Sigma-Aldrich] in 10 ml of 2-methyl-2-butanol [Kemira, Stockholm, Sweden]), as previously described (13). Some recipients received an intravenous injection of alloxan (75 mg/kg body wt; Sigma-Aldrich) 5 days before transplantation and had blood glucose concentrations >19.7 mmol/l (304 ± 1.3; n = 10) at the time of transplantation. These diabetic recipients received a transplant of 200–400 cultured islets to fully reverse the hyperglycemia. Upon retrieval of the transplanted islets 1 month posttransplantation, all had blood glucose concentrations <12 mmol/l (9.5 ± 0.6; n = 10). Blood from the cut tip of the tails was used to measure...
blood glucose concentrations by means of glucose reagent strips (MediSense Sverige, Sollentuna, Sweden).

Transmission electron microscopy. Some of the retrieved transplanted islets were prepared for and studied with transmission electron microscopy (TEM), as previously described (17). Briefly, the retrieved islets were fixed overnight in 2.5% (vol/vol) glutaraldehyde (Sigma-Aldrich) and 0.1 mol/l cacodylate buffer (Agar Scientific, Stansted, U.K.). After being washed in 0.1 mol/l cacodylate buffer, the islets were postfixed for 20 min in 1% (wt/vol) OsO4 dissolved in cacodylate buffer. A second wash in cacodylate buffer was followed by dehydration in graded series of ethanol, before the islets were finally embedded in Arag 100 Resin (Agar Scientific). Sections were cut, contrasted, and examined in a Hitachi H7100 transmission electron microscope (Hitachi, Tokyo, Japan) at 75 kV.

Scanning electron microscopy. Islets were fixed and postfixed as described above for TEM and thereafter washed in 0.1 mol/l cacodylate buffer and distilled water. The islets were mounted on poly-L-lysine–treated coverslips and dehydrated in a graded series of acetone by means of the critical-point drying method. After drying, the coverslips were mounted on holders and the islets were gold sputtered and then examined in a LEO 1530 field emission scanning electron microscope (LEO, Cambridge, U.K.) at an accelerating voltage of 5 kV.

Glucose- and theophylline-stimulated insulin release. Groups of 10 islets were transferred in triplicate to glass vials containing 250 μl of Krebs-Ringer bicarbonate buffer supplemented with 10 mmol/l HEPES (Sigma-Aldrich) and 2 mg/ml BSA (ICN Biomedicals, Aurora, OH; hereafter referred to as KRBH buffer). The KRBH buffer contained 1.67 mmol/l n-glucone at the first hour of incubation at 37°C (O2/CO2, 95:5). The medium was then removed and replaced by 250 μl of KRBH supplemented with 16.7 mmol/l glucose and incubated for a second hour. The medium was again removed, and the islets were, in some cases, incubated with 250 μl of KRBH supplemented with 16.7 mmol/l glucose and 5 mmol/l theophylline (Apotekbolaget, Gotenburg, Sweden) for a third hour. After retrieval of this medium, the islets were harvested, pooled in groups of 30, and homogenized by sonication in 200 μl of redistilled water. Two 50-μl aliquots of the aqueous homogenate were then used for DNA measurements by fluorophotometry (18). A fraction of the homogenate was mixed with acetic acid (0.18 mol/l HCl in 95% [vol/vol] ethanol) from which insulin was extracted overnight at 4°C. Insulin contents in incubation media and homogenates were determined by enzyme-linked immunosorbent assay (Mercodia, Uppsala, Sweden). In pilot experiments, the influence of neutral red on glucose-stimulated insulin release and insulin content was also evaluated. Triplicates of 10 islets were incubated at a high glucose concentration (16.7 mmol/l glucose + KRBH) with or without addition of 5 mg/ml neutral red for 1 h. Thereafter, islets and incubation medium were harvested and analyzed for insulin concentration as outlined above.

Glucose oxidation. Islet glucose oxidation rates were determined according to a previously described method (19). Briefly, triplicates of 10 islets were transferred to glass vials containing 100 μl of KRBH supplemented with 6.0 μmol/l [1-14C]glucose (Amersham-Pharmaciatech Biotech, Amersham, U.K.) and non-radioactive n-glucose to a final glucose concentration of 16.7 mmol/l glucose (specific radioactivity 0.5 μCi · mmol−1 · 1−1). After incubation for 90 min at 37°C (O2/CO2, 95:5), the oxidation was terminated by injection of 100 μl of 0.05 mmol/l antimycin A (Sigma-Aldrich) into the vials. 14CO2 generated by cell metabolism was released by the addition of 100 μl of 0.4 mmol/l NaHPO4 (pH 6.0) during a 120-min incubation. The radioactivity in the samples was then measured by liquid scintillation counting. In pilot experiments, the influence of 5 mg/ml neutral red on glucose oxidation rate was also evaluated.

Statistical analysis. All values are given as means ± SE. In each experiment, insulin secretion and glucose oxidation rates were calculated as a mean of the values obtained from the three individual incubation vials. These means were then considered as separate observations in the subsequent statistical analysis. Multiple comparisons between data were performed by using ANOVA (Statview; Abacus Concepts, Berkeley, CA) and, when appropriate, followed by Bonferroni post hoc test. Where only two experimental groups were compared, unpaired t test was used. P < 0.05 was considered to be statistically significant for all comparisons.

RESULTS
Identification of retrieved transplanted islets. Normally, 50–100 islets were retrieved from each processed liver. The identity of the retrieved transplanted islets was confirmed by intravital staining, immunohistochemistry, and electron microscopy. Retrieved islets were stained pink/red with the intravital staining neutral red, whereas surrounding hepatocytes remained unstained (Fig. 2A). A large number of cells staining positive with insulin anti-

FIG. 1. Schematic drawing of preparation of graft-bearing liver for perfusion and retrieval of islets. C, catheter; H, heart; I, inferior vena cava; L, liver; P, portal vein; S, superior vena cava. Perfusion was made in a retrograde direction through the liver (arrow).
bodies was found in sections of the retrieved islets prepared for light microscopy (Fig. 2B). In sections of the islets prepared for TEM, a normal islet ultrastructure with intact cells and secretory granules characteristic of β- and α-cells was observed (Fig. 2C). When examined by scanning electron microscopy, the retrieved islets were intact, but a few contaminating cells were found adherent to their surface (Fig. 2D and E).

**Insulin release and insulin content.** Insulin release from retrieved islets in response to stimulation with 1.67 mmol/l glucose, 16.7 mmol/l glucose, or 16.7 mmol/l glucose + 5 mmol/l theophylline was investigated either immediately after retrieval or after 3–4 days of culture. For comparative purposes, the corresponding insulin response from freshly isolated or cultured endogenous islets was examined (Fig. 3). Immediately after preparation,
isolated islets (theophylline for a third hour). Values are means ± SE. A: freshly isolated islets (n = 15) and freshly retrieved islets from normoglycemic (n = 8) and cured diabetic (n = 10) recipients. *P < 0.05 vs. freshly isolated islets; #P < 0.05 vs. corresponding islets exposed to 1.67 mmol/l glucose. B: isolated and cultured islets (n = 6) and cultured retrieved islets (n = 7). *P < 0.05 vs. isolated and cultured islets; #P < 0.05 vs. corresponding islets exposed to 1.67 mmol/l glucose.

FIG. 4. Insulin content in isolated nontransplanted islets (■) or intraportally transplanted islets retrieved from normoglycemic (□) or cured diabetic recipients (□). The experimental groups were freshly isolated islets (n = 11), freshly retrieved islets from normoglycemic (n = 9) or cured diabetic recipients (n = 9), isolated and cultured islets (n = 7), and cultured islets retrieved from normoglycemic recipients (n = 7). Values are expressed as means ± SE. *P < 0.05 vs. corresponding isolated nontransplanted islets; #P < 0.05 vs. corresponding freshly isolated islets.

islets retrieved from both normoglycemic and cured diabetic recipients released less insulin than endogenous control islets during all three conditions (Fig. 3A). After culture, the insulin response to 16.7 mmol/l glucose and 16.7 mmol/l glucose + theophylline remained markedly impaired in retrieved islets, whereas basal insulin release was similar to that seen from cultured control islets (Fig. 3B). Both freshly isolated and cultured control islets released more insulin when challenged with 16.7 mmol/l glucose or 16.7 mmol/l glucose + theophylline compared with when exposed to 1.67 mmol/l glucose (Fig. 3). Statistical significance, however, was not attained for freshly isolated islets challenged with 16.7 mmol/l glucose only. Retrieved islets responded poorly, both immediately after isolation and after culture, when stimulated with high glucose (Fig. 3). Only 16.7 mmol/l glucose + theophylline induced a slight but statistically significant increase in insulin release from the freshly retrieved islets.

Retrieved transplanted islets contained less insulin, both immediately after retrieval and after culture, compared with control islets (Fig. 4). However, although the insulin content of control islets was markedly decreased after culture, the insulin content of retrieved islets did not decrease any further. The insulin content of islets freshly retrieved from normoglycemic and cured diabetic recipients did not differ. In pilot experiments, the influence of the intravital stain neutral red on glucose-stimulated insulin release and islet insulin content was evaluated. Neither insulin release nor insulin content of islets was affected by this stain (10.8 ± 1.4 vs. 10.8 ± 1.9 ng insulin/10 islets and 640 ± 50 vs. 580 ± 130, for insulin release in response to 16.7 mmol/l glucose and insulin content of control and neutral red–exposed freshly isolated islets, respectively; n = 2). Repeated exposure to collagenase of isolated islets affected basal insulin release neither acutely (data not shown) nor after culture (0.7 ± 0.1 vs. 1.1 ± 0.5). Likewise, the insulin release response to 16.7 mmol/l glucose and that to 16.7 mmol/l glucose + theophylline was unaffected (7.5 ± 3.1 vs. 4.1 ± 0.6 and 37.7 ± 10.8 vs. 33.5 ± 4.5 for insulin release of control and collagenase-exposed islets, respectively; n = 8 in all groups). The insulin content of the isolated islets also was not affected by the repeated collagenase exposure (273 ± 30 vs. 287 ± 30, for control islets and collagenase-exposed cultured islets, respectively).

Glucose oxidation rate. When retrieved islets were exposed to 16.7 mmol/l glucose, the glucose oxidation rate was markedly lower than that of control islets, both immediately after retrieval and after culture (Fig. 5). The glucose oxidation rate was not affected by culture in either retrieved transplanted or control islets. Neither did the glucose oxidation rates of islets retrieved from normoglycemic and cured diabetic recipients differ. Addition of neutral red did not affect the glucose oxidation rate of freshly isolated control islets (219 ± 15 vs. 209 ± 51 pmol · 10 islets⁻¹ · 90 min⁻¹ for control and neutral red–exposed islets, respectively; n = 2). Repeated exposure to collagenase of isolated islets did not affect the glucose oxidation rate.
The major finding in the present study was that islets that were retrieved from the liver seemed to be functionally compromised. This may be due to their intrahepatic location per se, but there may be other explanations for the poor metabolic response of the retrieved islets. It can be argued that the decreased glucose-stimulated insulin release, insulin content, and glucose oxidation rate of retrieved islets compared with control islets merely reflect a lower amount of islet tissue in the former preparations. However, the DNA content of freshly isolated islets and retrieved islets was similar, which argues against this notion. It cannot be excluded, however, that some hepatocytes contaminated the transplanted islets when experiments with retrieved islets were performed, which may affect both the DNA content and the function of the islets. Great effort was taken to use the purest islets possible for the functional evaluations, and, in view of our morphologic findings, contaminating cells were sparse. Endothelial cells, which are more common in freshly isolated islets (23), might be another source of contamination. Hirshberg et al. (24) actually observed that freshly isolated islets from normoglycemic (23), might be another source of contamination. Hirshberg et al. (24) actually observed that freshly isolated islets that were transplanted into the liver become covered by an endothelial cell layer. However, we could not observe such cells on the retrieved islets. We also believe that after several days of free-floating culture of the retrieved islets, most contaminating cells have disappeared (cf. 23). Therefore, the consistently impaired islet function also after culture argues against the possibility that this is caused by the mere presence of contaminating cells.

Another potential explanation for the poor function of the retrieved islets is that the retrieval procedure in itself damages the islet cells. However, the same amount of collagenase (3.1 mg/ml) was used as that for isolating islets from the pancreas (12,25). Moreover, in separate in vitro experiments, we imitated the exposure of isolated islets to collagenase in vivo. However, there was no influence on glucose-stimulated insulin release, islet insulin content, or glucose oxidation rate. In further support of the view that the functional defects are inherent to the retrieved islets and not dependent on the retrieval procedure, the retrieved islets functioned poorly compared with nontransplanted control islets also after several days in culture.

Previously, islets that were implanted under the renal capsule were retrieved and examined (26). A decreased first phase of glucose-stimulated insulin release (27) and a chronically decreased insulin content compared with control islets (28) were observed. Such islets retrieved from the kidneys also display metabolic aberrations with increased nonoxidative metabolism (29), which is similar to our previous findings in vivo (30). In the present study, functional impairment seen in intraportal islets was very pronounced, suggesting that the implantation organ may be of importance. However, islets that were retrieved from implantation sites other than the liver were not investigated.

A problem in the immediate posttransplantation period may be that the liver parenchyma has an oxygen tension of 5–10 mmHg, which is markedly lower than what is seen in most other tissues, including the pancreas (31,32). This most likely reflects the high metabolic activity of the tissue and that ~60–70% of the blood supply to the liver constitutes venous blood from the portal vein. The latter, how-
ever, is unlikely to be of major importance for implanted islets after engraftment, because pancreatic islets that are transplanted intraportally into the liver become revascularized mainly from the hepatic artery (33). Because these islets have a decreased vascular density compared with endogenous islets (16), it is nevertheless likely that they are exposed to chronic hypoxia. This is confirmed by the findings that islets that are implanted beneath the liver capsule have low oxygen tension (5–10 mmHg) similar to that of islets that are implanted into the renal subcapsular space (32).

Despite physiological advantages of portal delivery of insulin compared with systemic delivery of the hormone, islets that are implanted intraportally have a lower cell proliferation rate and a decreased functional reserve compared with islets that are grafted into the kidney (13,34). Experimental studies (34) in rodents have also indicated a lower long-term rate of function of intraportally transplanted islets than islets that are implanted at other sites. This suggests that the liver as a site for islet implantation imposes considerable stress on the graft, leading to diminished survival and/ or impaired function of the endocrine cells. Some of this stress may be due to the new islet milieu with portal blood rich in glucose after meals and the proximity to glycogen-containing hepatocytes with their high gluconeogenetic capacity.

The present study was performed in the absence of specific immunologic responses to the graft because syngeneic transplantations were performed. An allogeneic setting might provide further site-specific challenges for transplanted islets. Thus, islet activation of resident liver macrophages with production of cytokines and free radicals, e.g., nitric oxide is known to be toxic to islet β-cells, may contribute to islet cell death and dysfunction at this implantation site (35–39).

We obtained similar results with islets that were freshly retrieved from both normoglycemic and cured diabetic recipients. The influence of an intact endogenous endocrine pancreas on the function of intraportally transplanted islets is previously largely unknown. However, studies (27,30,40) of islet grafts implanted beneath the renal capsule by means of microdialysis, perfusion, and perfusion experiments have shown that islet grafts become and remain functionally active despite the presence of endogenous islets.

It cannot be excluded that the function of the retrieved islets is not necessarily representative of all transplanted islets and that we have managed to isolate only a functionally deranged subpopulation of the islets. However, we recently observed that only ~65% of intraportally transplanted islets remain in the liver as intact islets (E. von Seth, G.M., P.-O.C., L.J., unpublished observation). This means that in the present study, ~75% of all remaining transplanted islets were retrieved and that although larger functional variations occurred in the retrieved islets than in control islets, they consistently had impaired function. In view of this, we consider that the observed findings are likely representative of intraportally implanted islets in general. Furthermore, the decreased glucose oxidation rates implicate a predominant mitochondrial dysfunction of the transplanted cells as a key mechanism behind the impaired function. It is interesting that other disturbances in the function of intraportally transplanted islets have also been described. Disturbed physiological regulation of glucose metabolism with increased C-peptide concentrations and insulin resistance occur, as well as defective glucagon response, during hypoglycemia (41–43). Taken together, there is ample evidence that the intraportal site confers several disadvantages for an optimal endocrine function of transplanted islets.

In summary, we have developed a novel technique for retrieval of intraportally transplanted islets and show that such islets have a pronounced decrease in glucose-stimulated insulin release and insulin content when compared with isolated, nontransplanted control islets. The retrieved islets also had a markedly lower glucose oxidation rate than control islets, suggesting the presence of mitochondrial dysfunction. The technique described herein offers unique possibilities to also study further the metabolic and functional changes, induced by the intraportal site, in transplanted pancreatic islets.

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