Original Article

Superiority of Small Islets in Human Islet Transplantation

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Many factors influence the outcome of islet transplantation. As islets in the early posttransplant setting are supplied with oxygen by diffusion only and are in a hypoxic state in the portal system, we tested whether small human islets are superior to large islets both in vitro and in vivo. We assessed insulin secretion of large and small islets and quantified cell death during hypoxic conditions simulating the intraportal transplant environment. In the clinical setting, we analyzed the influence of transplanted islet size on insulin production in patients with type 1 diabetes. Our results provide evidence that small islets are superior to large islets with regard to in vitro insulin secretion and show a higher survival rate during both normoxic and hypoxic culture. Islet volume after 48 h of hypoxic culture decreased to 25% compared with normoxic culture at 24 h due to a preferential loss of large islets. In human islet transplantation, the isolation index (islet volume as expressed in islet equivalents/islet number), or more simply the islet number, proved to be more reliable to predict stimulated C-peptide response compared with islet volume. Thus, islet size seems to be a key factor determining human islet transplantation outcome. *Diabetes* 56:594–603, 2007

The field of clinical islet transplantation has slowly evolved during the past 3 decades (1). The success achieved with the Edmonton protocol (2,3) established islet transplantation as an alternative to pancreas transplantation. This success was possible by incremental improvements (4), including better identification of optimal donor characteristics (5), superior organ retrieval techniques (6,7), minimization of cold ischemia times, improved isolation technique, and avoidance of immunosuppressive drugs, which impair islet function (2). The Edmonton trial (2,3) recognized transplanted islet volume as a key factor for predicting insulin independence. To be able to compare the volume of islets with different diameters and volumes, individual islets are mathematically converted to standard islet equivalents (IEQs) with a diameter of 150 μm (8). The Edmonton trial can be regarded as a dose-finding study for islet transplantation in patients with type 1 diabetes. It was concluded that a total of ~12,000 IEQs/kg recipient body wt were needed to achieve insulin independence. This corresponds roughly to the presumed islet volume in a normal pancreas (9). The functional capacity of transplanted islets, however, was only 20–40% of that in a nondiabetic person (3,10). These findings strongly suggest that only a small fraction of the transplanted islets successfully engraft or, alternatively, that insulin production and secretion is severely compromised. It is estimated that 50–70% of the transplanted islets will be lost in the immediate posttransplantation period (11), the immediate blood-mediated inflammatory response being a major contributor to this striking early posttransplantation islet loss (9,12–15). However, as summarized by Smith and Gale (4), the fate of islets in the immediate posttransplant period is not only determined by the immediate blood-mediated inflammatory response but also by the hypoxic state in the portal vein, by the energy status of the islets, and finally by the balance between pro- and antiapoptotic mediators, which will determine the ability of the islets to resist subsequent proapoptotic insults. Thus, despite successes in different areas, islets from multiple donors are normally required to achieve insulin independence.

One factor that has been neglected until now is the size of the transplanted islets. Normally, pancreatic islets have a dense glomerular-like capillary network that entails a blood perfusion that is 10 times higher than in the exocrine pancreas (16), resulting in a significantly higher oxygen tension (pO_2 40 vs. 30 mmHg). However, during the process of isolation and in vitro culture the islet vasculature dedifferentiates or degenerates, and immediately after transplantation the pancreatic islets are supplied with oxygen and nutrients solely by diffusion. Based on data derived from carcinoma cell clusters, it can be assumed that if oxygen tension at the periphery is 40 mmHg, the tension in the center of an islet with a radius of 100 μm will be 0 mmHg (17). Oxygen tension in the portal blood stream, however, is only 3–5 mmHg. Even 9–12 weeks after transplantation to the liver and arterial revascularization, the oxygen tension is much lower than in the native pancreas (5 vs. 40 mmHg) and even lower in recipients with diabetes (16).

As islets in the early posttransplant setting will be supplied with oxygen by diffusion only and are in a hypoxic state in the portal system, we tested the hypothesis that human islets

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IEQ, islet equivalent; TUNEL, terminal dUTP nick-end labeling.

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with a smaller diameter are superior to large islets both in vitro and in vivo. The aim of the present study was, therefore, first to compare in vitro insulin production of small and large islets and to quantify cell death (including apoptosis and necrosis independent from the mechanisms involved) under hypoxic culture conditions, which mimic the hypoxic environment of the transplantation site in the liver. Second, we assessed if the size of transplanted islets independently predicted the stimulated insulin secretion in the clinical setting of islet transplantation.

RESEARCH DESIGN AND METHODS

Chemicals were purchased from Fluka (Fluka Chemie, Buchs, Switzerland) and from Invitrogen (Life Technologies/Invitrogen, Basel, Switzerland). Insulin and C-peptide were measured by radioimmunoassays (Insulin-CT, IRMA C-PEP; CIS Bio International, Schering, Baar, Switzerland). The intra- and interassay variations were, respectively, 5.9 and 7.9% for insulin and 4.9 and 5.8% for C-peptide.

Islet isolation. Human pancreata were removed from brain-dead donors and stored in cold University of Wisconsin solution. Cold ischemia times ranged from 2 to 10 h. The islet isolation was performed as previously described (18). In brief, the duct was perfused in a controlled manner with 170 ml cold collagenase (Hanks' balanced salts [Invitrogen], 3 mg/ml collagenase [Liberase HI; Roche], 70 mmol/l HEPES [Invitrogen], and 10 mmol/l $CaCl_2$ [Fluka]). The islets were separated from the exocrine tissue by enzymatic and mechanical dissociation. The digested pancreatic tissue was top loaded on a continuous Ficoll gradient with a density from 1.077 to 1.100 (Biochrom, Berlin, Germany) and centrifuged in a refrigerated cell separation apheresis system (COBE 2991; Lakewood, CO). The purified islets were stained with dithizone, counted, and measured with an image analysis system (Saisam; Microvision Instruments, Evry, France) to determine islet yield. Islets were cultured in CMRL 1066 medium (5.5 mmol/l glucose) containing 10% FCS, 100 μg/ml streptomycin, 100 units/ml penicillin, 1 mmol/l sodium pyruvate, 2 mmol/l glutamax, and 2 mmol/l HEPES, hereafter referred as $CMRL_{compl}$. Static glucose stimulation was performed in triplicate aliquots with 100 islets per sample.

Islet size assessment. Islets were handpicked according to size into two groups: small islets were defined as islets with a diameter between 50 and 150 μm and large islets with a diameter between 150 and 300 μm. Islets were counted and the diameter of every islet measured by the image analysis system Saisam. Islet volume is always expressed as IEQs. For each sample, the total islet volume (IEQs) (8), islet number, and size distribution were assessed. The isolation index (islet volume as expressed in IEQs per islet number) is an indicator of the average islet size (e.g., an index <1 indicates that the average islet size is <150 μm and vice versa). Histological sections were analyzed with a Zeiss Axiolab microscope, a Zeiss AxioCam HRc camera, and the imaging software AxioVision 3.1 (Carl Zeiss, Feldbach, Switzerland). Area and diameter of islets were measured using the AxioVision 3.1 software.

Perifusion. The technique of perifusion has been described previously (19). In short, two groups of islets with different sizes, as described above, were handpicked. The islets were placed in a chamber of 300 μl, embedded in Sephadex G-10 (Pharmacia, Uppsala, Sweden), and mounted in a perifusion device, preheated to 37°C (Superafusion 1000, model SF-06; Brandel, Gaithersburg, MD). The islets were perfused with Krebs-Ringer-HEPES buffer (131 mmol/l NaCl, 4.8 mmol/l KCl, 1.2 mmol/l $CaCl_2$*$2H_2O$, 25 mmol/l HEPES, 1.2 mmol/l KH_2PO_4, and 1.2 mmol/l $MgSO_4$*$7H_2O$) containing 0.1% human serum albumin (Sigma) (Krebs-Ringer-HEPES buffer–human serum albumin) and glucose (Fluka Chemie) at a flow rate of 250 μl/min, and samples of 500 μl were collected. During the first 100 min, the islets were perifused with 2.8 mmol/l glucose, from 100 to 160 min with 20 mmol/l glucose, and from 160 to 220 min with 2.8 mmol/l glucose.

Stimulated insulin secretion was determined as integrated insulin secretion over the time period of high glucose. The amount of insulin secreted is calculated as area under the curve per IEQ in order to adjust for differences in islet volume. Area under the curve for insulin was calculated by using the trapezoidal rule. Basal secretion was calculated by multiplying the average insulin secretion during low glucose exposure just before raising the glucose concentration to 20 mmol/l. The insulin secretion stimulation index was calculated by dividing the amount of stimulated insulin by the amount of insulin produced at basal conditions.

Static glucose stimulation. Static glucose stimulation was performed in triplicates with 100 islets per sample. Islets were incubated for 30 min in $RPMI_{compl}$ (RPMI-1640 without glucose [11879; Life Technologies/Invitrogen]), 10% FCS, 100 μg/ml streptomycin, 2 mmol/l glutamax, 100 units/ml penicillin, 10 mmol/l HEPES (Life Technologies/Invitrogen) with 2.8 mmol/l D-(+)-glucose to equilibrate the islets, for 2 h in $RPMI_{compl}$ 2.8 mmol/l glucose, for 2 h in $RPMI_{compl}$ 20 mmol/l glucose, and again for 2 h in $RPMI_{compl}$ 2.8 mmol/l glucose. The amount of insulin secreted at high glucose (20 mmol/l) was divided by the amount at low glucose (2.8 mmol/l), and the mean insulin stimulation index was calculated.

Hypoxic cultivation. Human islets were cultivated over night in $CMRL_{compl}$ at standard cell culture conditions (37°C, 5% CO_2, 20% O_2). Islets of all sizes were cultivated in one dish. They were divided in four equal samples, placed in 35-mm diameter dishes, and incubated for 24 or 48 h either at normoxic (20% O_2) standard cell culture conditions or in a hypoxia chamber (Coy Laboratory Products, Grass Lake, MI) at hypoxic (1% O_2), but otherwise standard, cell culture conditions. Real-time oxygen measurements were performed in a hypoxic chamber (Coy Laboratory Products) at 7.6 mmHg (1%) oxygen tension with an Oxylite fluorescence-based, non–oxygen-consuming device (Oxford Optronix). The oxygen sensor has been placed on the bottom of a 35-mm Petri dish filled with 2 mm medium (Life Technologies CMRL). With 100 human islets, a steady-state oxygen tension of 5.9 ± 1.5 mmHg was reached after 12 h of hypoxic exposure. After incubation, islets were washed once in PBS and fixed and stained as described below.

Immunohistochemistry. Human islets were fixed in Bouin solution or Shandon Zinc Formula-Fixx (Anatomical Pathology International, Runcorn, Cheshire, U.K.) for 20 min either directly after isolation or after 1 to several days in culture. The fixed islets were embedded in 2% agarose in PBS, dehydrated, and paraffinated in a tissue processor Leica TP1020 (Leica Microsystems, Glattbrugg, Switzerland). Three-micrometer sections were cut on a microtome (Leica RM2255). Sections were deparaffinated in xylol and rehydrated in a descending series of ethanol (99, 95, 90, 80, 70%, H_2O, PBS), permeabilized for 5 min in methanol, and blocked for 30 min at room temperature with 10% normal goat serum in PBS. The sections were incubated with the first antibody (diluted from 1:50 to 1:1,000 in PBS/1% normal goat serum according to the antibody for 2 h at room temperature) and with the second antibody (diluted from 1:50 to 1:100 in PBS/1% normal goat serum, 10 ng/ml DAPI) for 2 h at room temperature. Antibodies used were monoclonal antibody anti-insulin (clone K36AC10; Sigma-Aldrich, St. Louis, MO) used at a 1:1,000 dilution, rabbit anti-glucagon (A0565; Dako, Carpinteria, CA) at a 1:50 dilution, goat anti-rabbit Cy3 at a 1:100 dilution, and goat anti-mouse fluorescein isothiocyanate at a 1:50 dilution (Jackson ImmunoResearch Laboratories, West Grove, PA). Terminal dUTP nick-end labeling (TUNEL) staining was done using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (S7100-KIT; Qbiogene, Basel, Switzerland), and nuclei were counterstained with hemalaun.

Quantification of cell death by hematoxylin and eosin histological analysis and TUNEL staining. Since both apoptosis and necrosis play a substantial role in the early posttransplant setting, hematoxylin and eosin staining was used to quantify cell death (including apoptosis and necrosis). The islet diameters (effective diffusion distance to islet core) were measured using Zeiss Axiovision software; analysis was performed using Adobe Photoshop software. Every cell on a cross-section of an islet was counted. Cells with nuclear hyperchromaticity, condensation, fragmentation, and/or hypereosinophilic and coarsely granular cytoplasm were scored as dead cells. To calculate a TUNEL index (positive cells/100 cells), all positive and negative nuclei were recorded. Counting was done blinded by two independent observers (R.Z. and P.K.). Islet volume was calculated considering islets mathematically as spheroids.

Clinical islet transplantation and definition of clinical outcome. Simultaneous islet-kidney transplantation and steroid-free immunosuppression was performed according to protocol that has been described previously (18). This protocol was approved by the ethics committee of the University Hospital Zurich, and written informed consent of patients was obtained. Since engraftment and complete vascularization of transplanted islets in the portal fields takes 3–6 months (20), we used the maximal stimulated C-peptide value obtained during a mixed-meal tolerance at 3 months posttransplant as the main clinical outcome measure in the multiple regression analysis. Since every islet isolation is composed of islets with different sizes, multiple islet transplantations would make analysis of the effect of islet size on insulin secretion impossible. Therefore, only patients with a single islet transplant and no need for steroid-based rejection therapy during this time period were included ($n = 7$).

Statistical analysis. Results are expressed as means ± SE. Data were analyzed with Statistica for Windows software (1997; Statsoft, Tulsa, OK). Groups were compared with the Student's t test when parameters were normally distributed, with Mann-Whitney U test when parameters were not normally distributed, and a χ^2 test for categorical variables. Kruskal-Wallis one-way ANOVA on ranks was used for multiple group comparisons versus control subjects for nominal parameters. $P < 0.05$ was considered significant. Using a linear regression, we analyzed the effect of the islet volume (IEQs), the islet number, and the islet

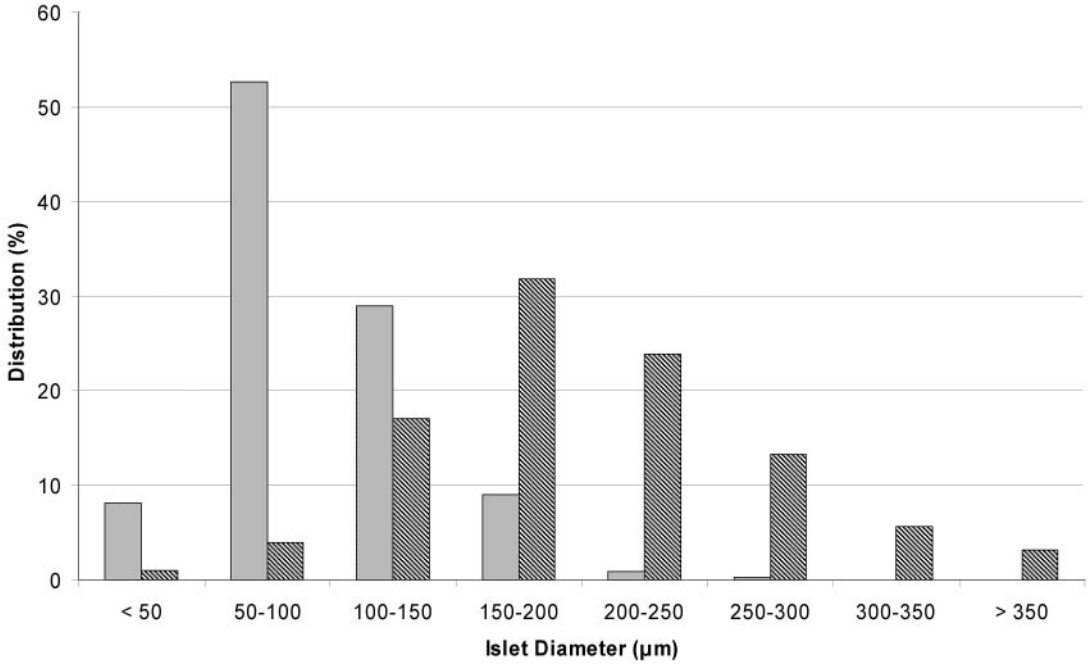

FIG. 1. Size distribution of islets used in perifusion experiments. Islets were handpicked according to size into two groups: small islets were defined as islets with a diameter between 50 and 150 µm and large islets with a diameter of 150–300 µm. ■, small islets; ▨, large islets.

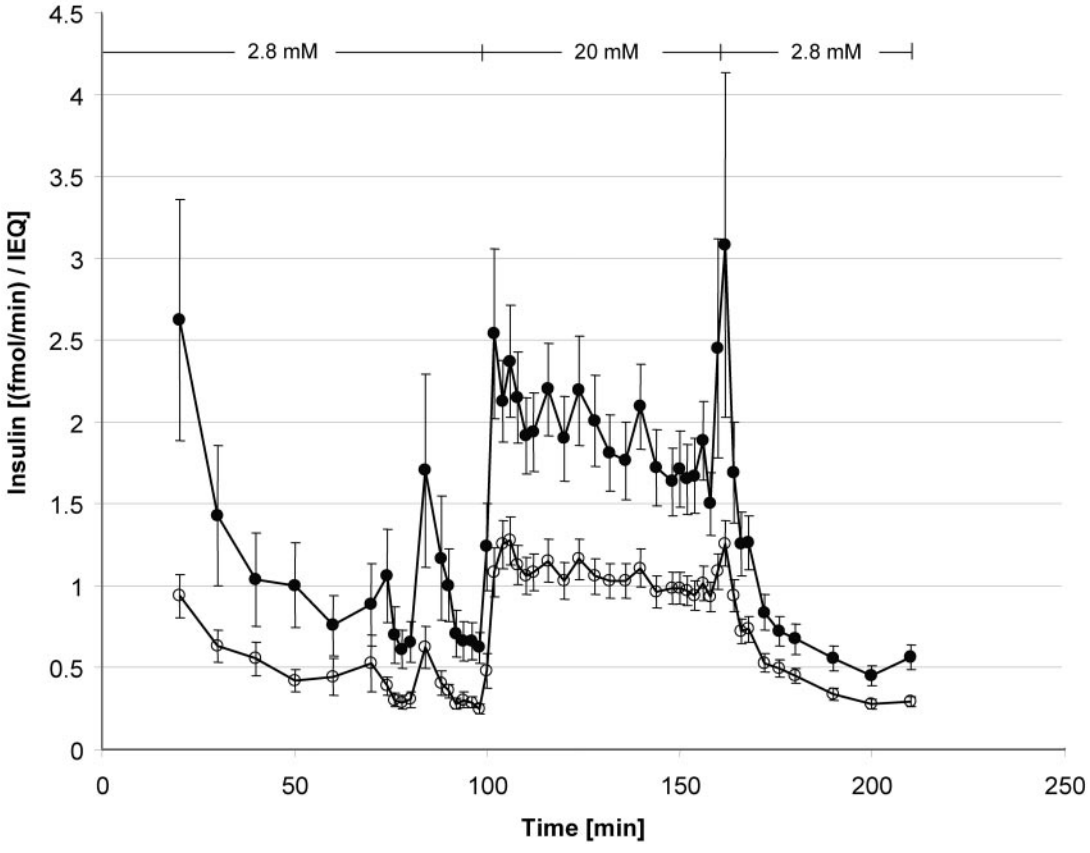

FIG. 2. Size-dependent insulin secretion corrected for islet volume (IEQ) in perifusion experiments. Islets were perifused with low (2.8 mmol/l) glucose from 0 to 100 min and with high glucose (20 mmol/l) from 100 to 160 min. Thereafter, islets were perifused again with low glucose solution. Data are means of 29 experiments (small versus large islets in each experiment). Islets with a small diameter (●) secrete almost double the amount of insulin as compared with large islets (○) assessed by the area under the curve ($P < 0.02$).

TABLE 1
Cell death during hypoxic and normoxic conditions according to islet size

Culture condition	Cell death (hematoxylin and eosin)		
	Islet size		
	<50 μm	50–100 μm	>100 μm
24 h normoxia	3.2 ± 0.6%	5.8 ± 0.8%	8.8 ± 1.8%
*n**	61	58	20
24 h hypoxia	9.7 ± 1.4%	12.4 ± 1.2%	17.5 ± 3.0%
n	54	48	8
48 h normoxia	5.0 ± 1.1%	6.8 ± 0.7%	6.6 ± 1.3%
n	40	67	16
48 h hypoxia	7.7 ± 1.7%	10.9 ± 1.3%	23.5 ± 11.3%
n	44	40	3

Data are means ± SE. *n = number of islets analyzed.

size (isolation index) on the maximally stimulated C-peptide response measured 3 months after simultaneous islet-kidney transplantation.

RESULTS

Islet isolations. Islets from 30 human pancreata were used for experiments. Means ± SE of the donor characteristics were age 53.0 ± 2.4 years, BMI 25.6 ± 0.7 kg/m^2, cold ischemia time 5:52 ± 0:19 h, total islet yield 281,894 ± 42,058 IEQs, islet number 204,700 ± 21,169, isolation index 1.3 ± 0.1, and stimulation index 4.1 ± 0.6. No difference in the donor BMI, age, static stimulation index, and cold ischemia time was seen between transplanted or not transplanted islets and between the islets used for the different groups of experiments. The total islet yield, islet number, and isolation index were significantly higher in islets used for human transplantation compared with nontransplanted islets (IEQs: 528,985 ± 76,229 vs. 188,034 ± 28,253, $P < 0.0001$; islet number: 316,571 ± 35,849 vs. 170,652 ± 20,927, $P = 0.001$; and isolation index: 1.8 ± 0.4 vs. 1.1 ± 0.1, $P = 0.004$).

In vitro experiments

Assessment of insulin secretion by perifusion experiments. The distribution of hand-picked islets (purity >90% by dithizone staining) used in the two groups (small and large islets) is given in Fig. 1. A total of 69 and 82% of the islets were in the desired size range. Insulin secretion adjusted for islet volume during perifusion was significantly different between large and small islets (area under the curve; $P < 0.02$; Fig. 2). Small islets with the same IEQs produced almost twice the amount of insulin compared with large islets in the basal state and during stimulation (stimulation: 1.9 ± 0.3 vs. 1.0 ± 0.1 fmol/[min × IEQs]); therefore, the insulin secretion stimulation index was not significantly different between the two groups (4.1 ± 0.5 vs. 5.3 ± 0.7, $P = 0.17$).

Cellular and morphological characteristics of islets and quantification of cell death (including apoptosis and necrosis). For normoxic and hypoxic conditions, cross-sections of 459 islets with 19,242 individual cells of eight different islet donors were analyzed, and distribution of glucagon- and insulin-positive cells was quantified by

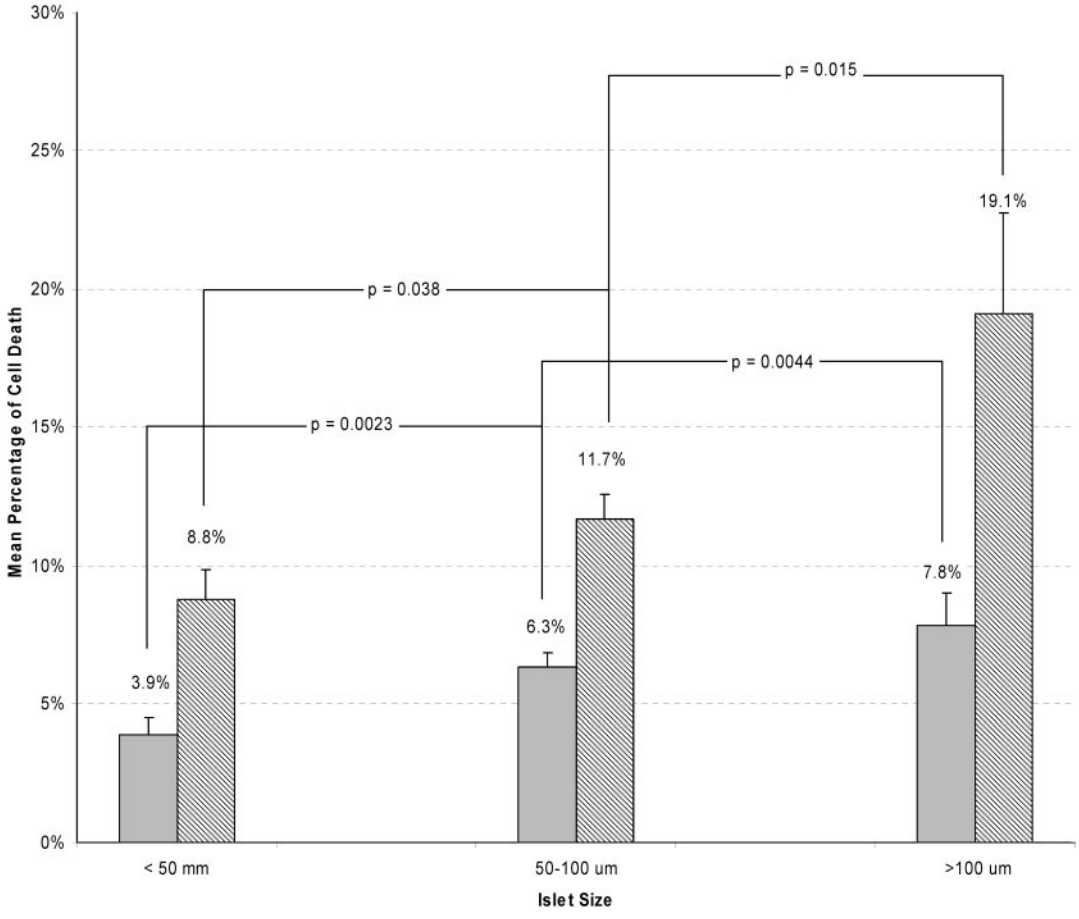

FIG. 3. Size- and hypoxia-dependent quantification of cell death in hematoxylin and eosin staining. At normoxic culture conditions cell death significantly increased in larger islets. This effect was almost doubled when hypoxia was applied. ▨, normoxia (24- and 48-h culture); ▧, hypoxia.

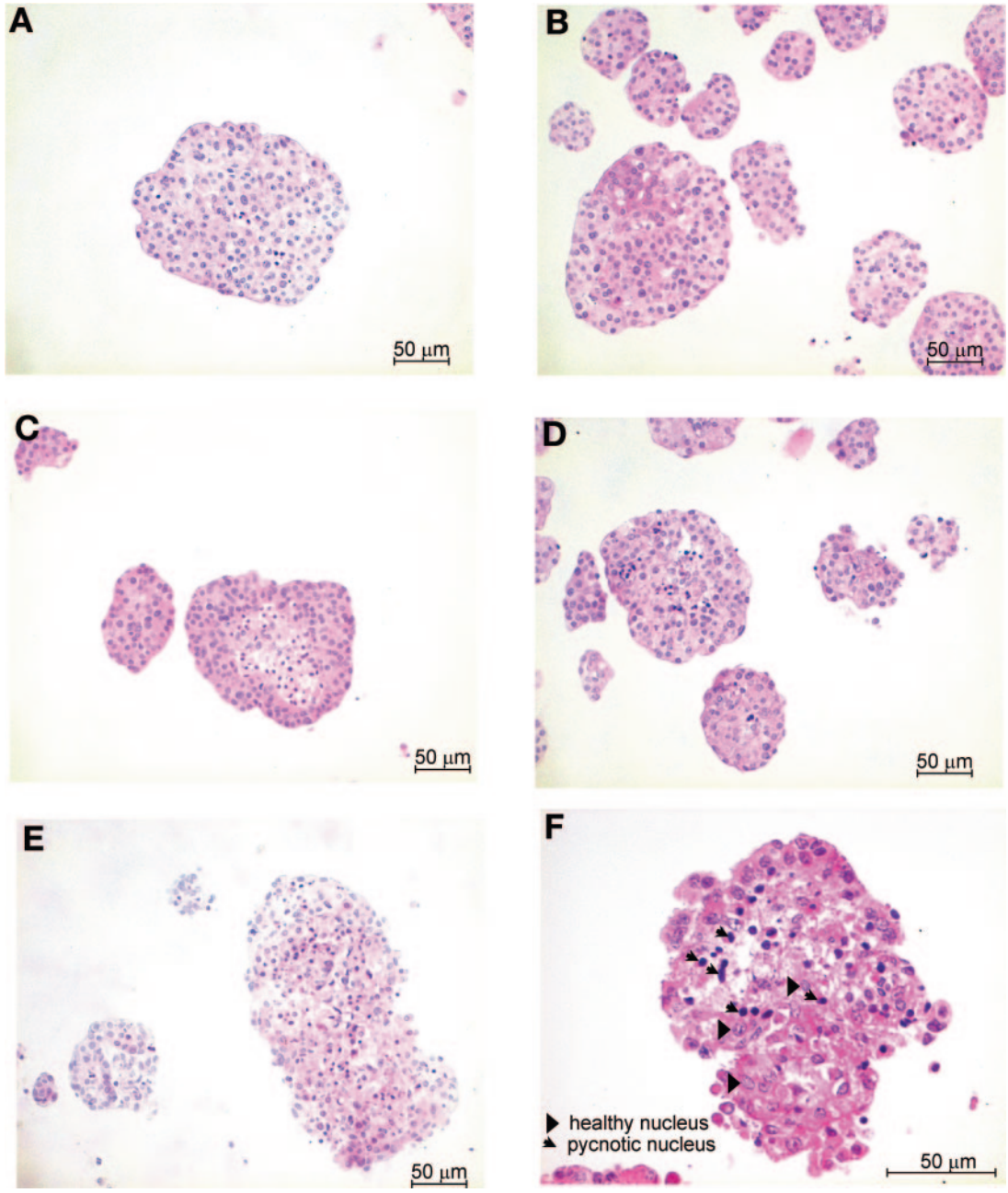

FIG. 4. Islet morphology at normoxic or hypoxic culture conditions. Isolated human islets, stained with hematoxylin and eosin. Islets were cultivated at normoxic conditions for 24 h (*A*) and 48 h (*B*) and at hypoxic conditions for 24 h (*C*, *E*, and *F*) and 48 h (*D*). An enlarged representative section for healthy and apoptotic nuclei and necrotic areas is shown in (*F*). Arrowhead, healthy nuclei; arrow, condensed, pycnotic nuclei. Islets cultivated under normoxic conditions (*A* and *B*) show less pycnotic dark nuclei and have a more intact structure then islets under hypoxic conditions (*C*–*F*), which show apoptotic/necrotic cores, of which the majority is preferentially found in the core of large islets.

immunohistochemistry. Cell death was evaluated with hematoxylin and eosin staining (Table 1).

Normoxia. The percentage of glucagon- and insulin-positive cells in relation to total cell number in the size range of 0–50 μm was 11 ± 3 and 76 ± 2%, respectively; in the range 50–100 μm 10 ± 1 and 65 ± 2%, respectively; and >100 μm 14 ± 3 and 50 ± 4%, respectively. The percentage of glucagon-positive cells was not significantly different between islet sizes, whereas the percentage of insulin-positive cells was significantly different between the size range 0–50 and >100 μm ($P < 0.0001$) and between 0–50 and 50–100 μm ($P = 0.0004$).

In hematoxylin and eosin staining at normoxic conditions, the number of pycnotic nuclei clearly increased with islet size (Figs. 3 and 4). In the size range of 0–50 μm, 3.9 ± 0.6% of cells were pycnotic compared with 6.3 ± 0.5% of cells in the range 50–100 μm ($P = 0.0023$) and 7.8 ± 1.2% of cells in the range >100 μm ($P = 0.0044$ vs. islets 50–100 μm). Apoptosis as assessed by TUNEL staining contributes to cell death, with 12.8–23.6% of the cells being TUNEL positive independent of islet size. The percentage of insulin-positive cells undergoing apoptosis as assessed by TUNEL and insulin costaining was 18.5 ± 2.9%, whereas the number of insulin-negative cells undergoing apoptosis was 12.1 ± 2.1% ($P = 0.078$) independent from islet size.

Hypoxia. The percentage of glucagon- and insulin-positive cells in the size range of 0–50 μm was 14 ± 2% and 70 ± 3%,

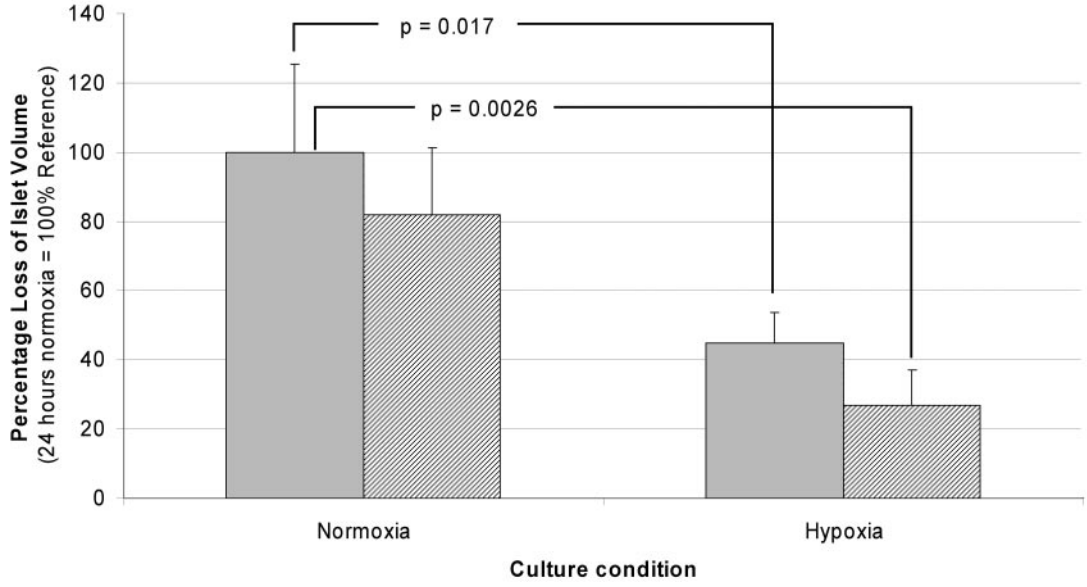

FIG. 5. Loss of islet volume during normoxic and hypoxic culture conditions. Twenty-four hour normoxic incubation was used as reference (100%) and compared with 48 h of normoxia and to 24 and 48 h of hypoxia simulating transplant environment in the portal vein (1% O_2). ■, 24-h culture; ▨, 48-h culture.

respectively; in the range 50–100 μm, 10 ± 1% and 53 ± 3%, respectively; and >100 μm, 11 ± 2% and 38 ± 8%, respectively. The percentage of glucagon-positive cells was not significantly different between the islet sizes, whereas the percentage of insulin-positive cells was significantly different between the size range 0–50 and >100 μm ($P < 0.002$) and between 0–50 and 50–100 μm ($P < 0.0001$).

The number of pycnotic nuclei clearly increased under hypoxic conditions: 8.8 ± 1.1% of nuclei were pycnotic in the size range of 0–50 μm compared with 11.7 ± 0.9% in the size range 50–100 μm ($P = 0.038$) and 19.1 ± 3.5% in islets >100 μm ($P = 0.015$ vs. islets 50–100 μm) (Fig. 3). A total of 14.1–24.5% of cells were TUNEL positive, with no difference being observed according to islet size. The percentage of insulin-positive cells undergoing apoptosis as assessed by TUNEL and insulin costaining was 26.6 ± 5.0%, whereas the number of insulin-negative cells undergoing apoptosis was 24.8 ± 3.5% (NS) independent from islet size.

Loss of islet number during hypoxic and normoxic culture conditions. After 24 h of hypoxia 21% islets were lost compared with normoxic culture conditions, and after 48 h of hypoxia 29% of islets were lost compared with 48 h of normoxic culture conditions. Thus, there was a mean 25% loss of islet number due to hypoxia.

Loss of islet volume during hypoxic and normoxic culture conditions. The islet volume after 24 h of normoxia as assessed by hematoxylin and eosin staining was 4,803,786 ± 1,214,077 μm^3. This value was used as reference (100%) and compared with the value at 48 h of normoxia and at 24 and 48 h of hypoxia. The islet volume after 48 h of normoxia decreased to 3,947,276 ± 930,960 μm^3. This decrease was dramatically pronounced by hypoxia. Islet volume decreased after a 48-h culture in hypoxic conditions to 1,312,861 ± 479,415 μm^3 compared with 2,171,424 ± 412,254 μm^3 after 24 h hypoxia (Fig. 5). The difference between hypoxic and normoxic conditions at 24 h ($P = 0.017$) and 48 h ($P = 0.0026$) was highly significant. The percentage of islets with a diameter of >100 μm did not change from 24 to 48 h of normoxia (14.4 vs. 14.2%), but there was a massive decrease in these large islets under hypoxic conditions from 7.3% after 24 h to 3.4% after 48 h of hypoxia, whereas the number of small (<50 μm) and medium (50–100 μm) islets did not change significantly. Hence, we assume that a large proportion of the islet volume loss can predominantly be attributed to large islets and not that large islets are fragmented into small islets.

In vivo data

Correlation of transplanted islet size with C-peptide production in patients with type 1 diabetes after simultaneous islet-kidney transplantation. The donor and recipient characteristics as well as the transplanted islet volume, islet number, and isolation index are given in Table 2. All recipients had end-stage renal failure and were listed for a simultaneous islet-kidney transplant.

The mean islet size or the isolation index (IEQs/islet number) proved to be the best predictor for the C-peptide response per islet volume per kilogram of recipient weight (Fig. 6*C*). Most of the variability (89%) could be accounted for by this predictor ($R^2 = 0.89$). The IEQ per kilogram of recipient weight was not able to predict stimulated C-peptide response (Fig. 6*A*), whereas the islet number per kilogram of recipient weight explained 84% of the stimulated C-peptide response (Fig. 6*B*).

DISCUSSION

Many factors, such as better identification of optimal donor characteristics (5), superior organ retrieval techniques (6,7), minimization of cold ischemia times, improved isolation technique, and avoidance of immunosuppressive drugs, which impair islet function (4), have been identified that influence the outcome of islet isolation and clinical islet transplantation (3,5,21–23). The fact that the functional capacity of transplanted islets in patients with type 1 diabetes is only about a third of the capacity of a person without diabetes indicates that two-thirds of transplanted islets are either lost or show a reduced function. To determine the amount of transplanted β-cells, IEQs are

TABLE 2
Characteristics of donor, recipient, transplanted islets, and outcome data

Recipient	Donor data				Recipient data						
	Age (years)	BMI (kg/m^2)	Cold ischemia time (h)	Pancreas weight (g)	Age (years)	Weight (kg)	IEQ total	IEQs/kg	Islet number	Islet number/kg	Isolation index	C-peptide stimulated (pmol/l)
1	43	25.9	7:22	68	61	68	425,505	6,257	243,000	3,574	1.75	1,120
2	62	26.7	5:56	77	43	53	688,140	12,984	419,000	7,906	1.64	2,000
3	62	25.0	6:22	108	39	72	241,338	3,352	301,000	4,181	0.80	850
4	39	22.4	5:30	81	72	71	825,389	11,625	250,000	3,521	3.30	870
5	57	23.4	6:37	101	57	46	639,783	13,908	232,000	5,043	2.76	1,000
6	40	26.2	8:35	85	59	75	511,349	6,818	294,000	3,920	1.74	710
7	64	29.4	2:54	115	53	74	371,389	5,019	477,000	6,446	0.78	1,480
Means ± SE	52.4 ± 4.2	25.6 ± 0.9	6:10 ± 0:40	90.6 ± 6.6	54.9 ± 4.2	65.6± 4.3	528,985 ± 76,229	8,566 ± 1,585	316,571 ± 35,849	4,941 ± 628	1.8 ± 0.4	1,147 ± 170

calculated depending on the number and diameter of the islets; one IEQ corresponding to a virtual islet of 150 μm diameter (8).

By reporting islet transplantation results in IEQs, one important factor for posttransplant islet survival and clinical outcome has been neglected, namely the size of the islets. Here, we provide in vitro evidence, both at the functional and at the morphological level, as well as clinical data that islet size may be a crucial determinant of islet function and survival. As shown by the perifusion experiments, insulin secretion, but not the stimulation index, is greatly influenced by islet size. When normalized to IEQs, basal and stimulated insulin secretion was twice as high in small islets compared with large islets. Indeed, MacGregor et al. (24) found that in rat islets the insulin secretion is proportional to the oxygen consumption and that in smaller islets the oxygen consumption and insulin secretion are significantly higher.

In culture and after transplantation, the oxygen supply of islets and nutrients depends solely on diffusion. The pO_2 level may be sufficiently high throughout the transplanted islets to maintain cell viability, but there is insufficient oxygen to support substantial insulin production and secretion (16). Papas et al. (25) showed that insulin secretion was gradually affected with pO_2 levels <7mmHg. In perifused cultured pancreatic islets, insulin secretion decreased by 50%, when pO_2 was lowered to 10 mmHg in the perifusate (26). If oxygen supply depends on diffusion, then the islet radius (i.e., the diffusion distance) is the determinant factor for pO_2 levels in the center of the islets. Not only oxygen supply but also a reduced nutrient supply might be a limiting factor and contribution to central necrosis (27). It has been shown that glucose concentration is lower in the center of large compared with small islets (28). Thus, due to a shorter diffusion distance, oxygen and nutrient supply is better in smaller islets, which allows for a higher insulin production. The functional data that small islets perform better than large islets are further supported by our morphological findings. At normoxic conditions small islets contain 26% more functional insulin-positive cells compared with large islets, whereas the percentage of glucagon-positive cells is not different between small and large islets. This might explain a part of the difference in insulin production between small and large human islets.

Islet cell death was strongly dependent on islet size and oxygen tension. The percentage of cell death as assessed by hematoxylin and eosin morphology doubled during hypoxic compared with normoxic culture conditions and doubled in islets with a diameter of >100 μm compared with islets <50 μm (Fig. 3). Whereas the percentage of glucagon-positive cells did not decrease in islets during hypoxia, there was a tendency toward a larger decrease of insulin-positive cells in large compared with small islets (−12 vs. −6%).

The overall 25% loss of islet number between normoxic and hypoxic culture conditions gives only a hint at the real islet volume loss, because the number of large islets (>100 μm) is almost identical after 24 and 48 h of normoxia (14.4 and 14.2%), whereas after 24 h of hypoxia there is only half the number of large islets (7.3%) left and after 48 h of hypoxia only a quarter of the number (3.4%). Considering that 19% of islet number in this size category (>100 μm) comprises 78% of the total islet volume of the pancreas (9), this 11% decrease of the large islets is responsible for a massive drop in the total islet volume (Fig. 5).

The fact that up to 16% of cells in islets were TUNEL

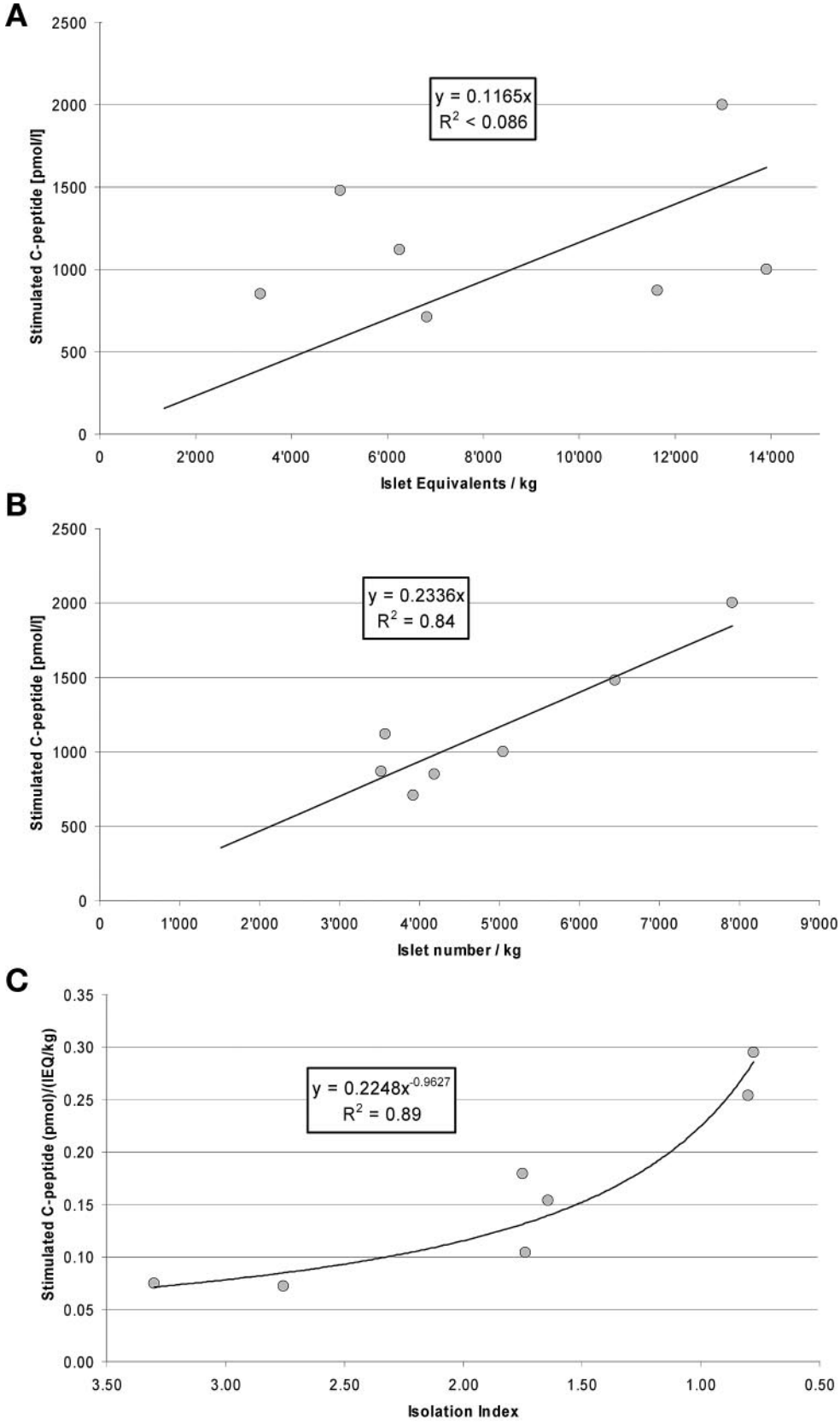

FIG. 6. Dependence of insulin secretion on transplanted islet volume, islet number, and islet size. Correlation of clinical outcome of islet transplantation as assessed by stimulated C-peptide production with the transplanted islet volume as defined by IEQs per kilogram of recipient weight (*A*), the transplanted islet number per kilogram of recipient weight (*B*), and the isolation index (*C*). Since insulin production is highly dependent on islet volume and the isolation index (IEQs/islet number) is a parameter of islet size only and not of islet volume, the stimulated C-peptide has to be corrected for the transplanted islet volume. The isolation index proved to be the best predictor for transplantation outcome (R^2 = 0.89).

positive during both normoxic and hypoxic conditions indicates that apoptosis is one of the mechanisms involved, possibly by hypoxia-inducible factor-1α signaling (29). However, as there was no correlation between TUNEL positivity and islet volume, mechanisms other than apoptosis, such as necrosis, must additionally be involved (27).

Several mechanisms might explain why large islets are more prone to cell death than small islets. After the isolation process, islets are no longer attached to the vascular system until revascularization takes place 7–10 days after transplantation (30). As reported by Carlsson et al. (16), transplanted islets have a pO_2 level of only 3–8 mmHg independent of the implantation site even after full revascularization. To simulate the oxygen tension in the liver, which is ~3–5 mmHg (16), we incubated the human islets in a hypoxic chamber with 1% oxygen concentration, which corresponds to the above-mentioned oxygen tension in the liver (as demonstrated in experiments).

To assess a possible role of islet size in the clinical setting of islet transplantation we compared islet function as defined by C-peptide production in relation to islet volume in a series of islet-transplanted patients. In these patients, there was a large variance of stimulated C-peptide response in relation to the transplanted islet volume (IEQs) (Fig. 6*A*). By using the islet number, however, 84% of the stimulated C-peptide response could be explained. Correction for islet size by use of the isolation index (IEQs/islet number) proved to be the best predictor for the C-peptide response per islet volume (Fig. 6*C*), with 89% of the variability being accounted for by this parameter. Although the number of patients used for the multiple regression analysis is low, the correlation between isolation index and C-peptide response seems to be quite robust. Our findings have to be confirmed in larger series of islet transplantations, preferably in patients who received islets from a single pancreas donor.

The patient with the second smallest islets (i.e., isolation index of 0.80) received only 3,351 IEQs/kg recipient body wt and 4,181 islets/kg recipient body wt, respectively. This patient had the identical maximal C-peptide response (850 pmol/l) as a patient who received much larger islets (isolation index of 3.3: 11,544 IEQs/kg and 3,497 islets/kg recipient body wt and a C-peptide response of 870 pmol/l). In other words, with an isolation index of 3.3 a 2.6-times higher transplanted islet volume (IEQs) with large islets was required to achieve the same insulin secretion as obtained by transplantation of small islets (Fig. 6*C*).

Our data are in line with recent findings in a rat model. MacGregor et al. (24) demonstrated that when a marginal islet volume of large islets was transplanted into diabetic rats, all recipients failed to achieve normoglycemia, whereas 80% of the transplantations using small islets were successful. Furthermore, single-donor insulin independence has been achieved successfully with younger pancreas donors (31), and isolated islets of younger donor pancreata showed a better insulin secretion in vivo and in vitro (32). In accordance with our results, these transplanted islets have been small (isolation index <1.0) (32). The necessary number of IEQs per kilogram of recipient weight to achieve insulin independence shows a large variation in clinical practice. An isolation with a small isolation index has most likely a smaller islet volume (IEQs) than an isolation with larger islets, because small islets contribute only very little to the total islet volume (volume ~ diameter^3). It is known that 80% of the total islet number is <100 μm in the human pancreas, but these islets comprise only 22% of the total volume, whereas only 5% of islets are >150 μm, but these islets comprise 47% of the total volume (9).

Our data suggest that in the setting of human islet transplantation, islet size may be a key factor that determines transplantation outcome. We therefore propose to use the isolation index as a correction factor for the traditionally reported islet volume in IEQs by using the following equation: stimulated C-peptide = IEQs/kg × 0.2248 × isolation index$^{-0.9627}$ as shown in Fig. 6*C*. Alternatively, and more simply, one could rely more on islet number (islet size >50 μm) instead of IEQs, with only a minimal loss in prediction power. Based on our small patient series, a minimal islet number of ~7,000 islets/kg recipient wt would be required to become insulin independent.

In the future, more emphasis has to be put on transplanting preferentially smaller islets. Based on our experiments and diffusion experiments in tumor cells (17), we suggest an optimal islet size of 50–100 μm, or more generally spoken, the smaller the islet the higher the chance for survival in a hypoxic milieu. By using islet isolations with an islet volume, which so far has been considered insufficient for a good clinical outcome, but with an adequate islet number, more islet isolations could now be used for transplantation. This would be a contribution to reduce the burden of organ donor shortage.

In conclusion, implementation of our findings might result in higher survival rate of islets, achievement of better islet transplantation outcomes, and economic savings by increasing the percentage of transplantable islet isolations.

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REFERENCES

1. Ricordi C, Inverardi L, Kenyon NS, Goss J, Bertuzzi F, Alejandro R: Requirements for success in clinical islet transplantation. *Transplantation* 79:1298–1300, 2005
2. Shapiro JA, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV: Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* 343:230–238, 2000
3. Ryan EA, Lakey JR, Rajotte RV, Korbutt GS, Kin T, Imes S, Rabinovitch A, Elliott JF, Bigam D, Kneteman NM, Warnock GL, Larsen I, Shapiro AM: Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol. *Diabetes* 50:710–719, 2001
4. Smith RM, Gale EAM: Survival of the fittest? Natural selection in islet transplantation. *Transplantation* 79:1301–1303, 2005
5. Lakey JR, Warnock GL, Rajotte RV, Suarez-Alamazor ME, Ao Z, Shapiro AMJ, Kneteman NM: Variables in organ donors that affect the recovery of human islets of Langerhans. *Transplantation* 61:1047–1053, 1996
6. Lakey JR, Tsujimura T, Shapiro AM, Kuroda Y: Preservation of the human pancreas before islet isolation using a two-layer (UW solution-perfluorochemical) cold storage method. *Transplantation* 74:1809–1811, 2002
7. Ricordi C, Fraker C, Szust J, Al Abdullah I, Poggioli R, Kirlew T, Khan A, Alejandro R: Improved human islet isolation outcome from marginal donors following addition of oxygenated perfluorocarbon to the cold-storage solution. *Transplantation* 75:1524–1527, 2003
8. Ricordi C, Gray DW, Hering BJ, Kaufman DB, Warnock GL, Kneteman NM, Lake SP, London NJ, Socci C, Alejandro R, et al: Islet isolation assessment in man and large animals. *Acta Diabetol Lat* 27:185–195, 1990

9. Korsgren O, Nilsson B, Berne C, Felldin M, Foss A, Kallen R, Lundgren T, Salmela K, Tibell A, Tufveson G: Current status of clinical islet transplantation. *Transplantation* 79:1289–1293, 2005
10. Ryan EA, Lakey JR, Paty BW, Imes S, Korbutt GS, Kneteman NM, Bigam D, Rajotte RV, Shapiro AM: Successful islet transplantation: continued insulin reserve provides long-term glycemic control. *Diabetes* 51:2148–2157, 2002
11. Davalli AM, Ogawa Y, Ricordi C, Scharp DW, Bonner-Weir S, Weir GC: A selective decrease in the beta cell mass of human islets transplanted into diabetic nude mice. *Transplantation* 59:817–820, 1995
12. Bennet W, Sundberg B, Groth CG, Brendel MD, Brandhorst D, Brandhorst H, Bretzel RG, Elgue G, Larsson R, Nilsson B, Korsgren O: Incompatibility between human blood and isolated islets of Langerhans: a finding with implications for clinical intraportal islet transplantation? *Diabetes* 48: 1907–1914, 1999
13. Johansson U, Olsson A, Gabrielsson S, Nilsson B, Korsgren O: Inflammatory mediators expressed in human islets of Langerhans: implications for islet transplantation. *Biochem Biophys Res Commun* 308:474–479, 2003
14. Moberg L, Johansson H, Lukinius A, Berne C, Foss A, Kallen R, Ostraat O, Salmela K, Tibell A, Tufveson G, Elgue G, Ekdahl KN, Korsgren O, Nilsson B: Production of tissue factor by pancreatic islet cells as a trigger of detrimental thrombotic reactions in clinical islet transplantation. *Lancet* 360:2039–2045, 2002
15. Ozmen L, Ekdahl KN, Elgue G, Larsson R, Korsgren O, Nilsson B: Inhibition of thrombin abrogates the instant blood-mediated inflammatory reaction triggered by isolated human islets: possible application of the thrombin inhibitor melagatran in clinical islet transplantation. *Diabetes* 51:1779–1784, 2002
16. Carlsson PO, Palm F, Andersson A, Liss P: Markedly decreased oxygen tension in transplanted rat pancreatic islets irrespective of the implantation site. *Diabetes* 50:489–495, 2001
17. Tannock IF: Oxygen diffusion and distribution of cellular radiosensitivity in tumors. *Br J Radiol* 45:515–524, 1972
18. Lehmann R, Weber M, Berthold P, Zuellig R, Pfammatter T, Maedler K, Donath MY, Ambuehl P, Demartines N, Clavien PA, Spinas GA: Successful simultaneous islet-kidney transplantation using a steroid-free immunosuppression: two-year follow-up. *Am J Transplant* 4:1117–1123, 2004
19. Spinas GA, Laffranchi R, Francoys I, David I, Richter C, Reinecke M: The early phase of glucose-stimulated insulin secretion requires nitric oxide. *Diabetologia* 41:292–299, 1998
20. Rooth P, Dawidson I, Lafferty K, Diller K, Armstrong J, Pratt P, Simonsen R, Taljedal IB: Prevention of detrimental effect of cyclosporin A on vascular ingrowth of transplanted pancreatic islets with verapamil. *Diabetes* 38 (Suppl. 1):202–205, 1989
21. Lakey JR, Warnock GL, Shapiro AM, Korbutt GS, Ao Z, Kneteman NM, Rajotte RV: Intraductal collagenase delivery into the human pancreas using syringe loading or controlled perfusion. *Cell Transplant* 8:285–292, 1999
22. Benhamou PY, Oberholzer J, Toso C, Kessler L, Penfornis A, Bayle F, Thivolet C, Martin X, Ris F, Badet L, Colin C, Morel P: Human islet transplantation network for the treatment of type I diabetes: first data from the Swiss-French GRAGIL consortium (1999–2000). Groupe de Recherche Rhin Rhjne Alpes Geneve pour la transplantation d'Ilots de Langerhans. *Diabetologia* 44:859–864, 2001
23. Ris F, Toso C, Veith FU, Majno P, Morel P, Oberholzer J: Are criteria for islet and pancreas donors sufficiently different to minimize competition? *Am J Transplant* 4:763–766, 2004
24. MacGregor RR, Williams SJ, Tong PY, Kover K, Moore WV, Stehno-Bittel L: 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
25. Papas KK, Long RC Jr, Constantinidis I, Sambanis A: Effects of oxygen on metabolic and secretory activities of beta TC3 cells. *Biochim Biophys Acta* 1291:163–166, 1996
26. Dionne KE, Colton CK, Yarmush ML: Effect of hypoxia on insulin secretion by isolated rat and canine islets of Langerhans. *Diabetes* 42:12–21, 1993
27. Giuliani M, Moritz W, Bodmer E, Dindo D, Kugelmeier P, Lehmann R, Gassmann M, Groscurth P, Weber M: Central necrosis in isolated hypoxic human pancreatic islets: evidence for postisolation ischemia. *Cell Transplant* 14:67–76, 2005
28. Kauri LM, Jung SK, Kennedy RT: Direct measurement of glucose gradients and mass transport within islets of Langerhans. *Biochem Biophys Res Commun* 304:371–377, 2003
29. Moritz W, Meier F, Stroka D, Giuliani M, Kugelmeier P, Nett PC, Lehmann R, Candinas D, Gassmann M, Weber M: Apoptosis in hypoxic human pancreatic islets correlates with HIF-1 alpha expression. *FASEB J* 16:745–747, 2002
30. Menger MD, Jaeger S, Walter P, Feifel G, Hammersen F, Messmer K: Angiogenesis and hemodynamics of microvasculature of transplanted islets of Langerhans. *Diabetes* 38 (Suppl. 1):199–201, 1989
31. Hering BJ, Kandaswamy R, Ansite JD, Eckman PM, Nakano M, Sawada T, Matsumoto I, Ihm SH, Zhang HJ, Parkey J, Hunter DW, Sutherland DER: Single-donor, marginal-dose islet transplantation in patients with type 1 diabetes. *JAMA* 293:830–835, 2005
32. Ihm SH, Matsumoto I, Sawada T, Nakano M, Zhang HJ, Ansite JD, Sutherland DER, Hering BJ: Effect of donor age on function of isolated human islets. *Diabetes* 55:1361–1368, 2006