β-Cell Death and Mass in Syngeneically Transplanted Islets Exposed to Short- and Long-Term Hyperglycemia

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We studied the effects of hyperglycemia on β-cell death and mass in syngeneically transplanted islets. Six groups of STZ-induced diabetic C57BL/6 mice were transplanted with 100 syngeneic islets, an insufficient β-cell mass to restore normoglycemia. Groups 1, 2, and 3 remained hyperglycemic throughout the study. Groups 4, 5, and 6 were treated with insulin from day 7 before transplantation to day 10 after transplantation. After insulin discontinuation, group 6 mice achieved definitive normoglycemia. Grafts were harvested at 3 (groups 1 and 4), 10 (groups 2 and 5), and 30 (groups 3 and 6) days after transplantation. On day 3, the initially transplanted β-cell mass (0.13 ± 0.01 mg) was dramatically and similarly reduced in the hyperglycemic and insulin-treated groups (group 1: 0.048 ± 0.002 mg; group 4: 0.046 ± 0.007 mg; $P < 0.001$). Extensive islet necrosis (group 1: 30.7%; group 4: 26.8%) and increased β-cell apoptosis (group 1: 0.30 ± 0.05%; group 4: 0.42 ± 0.07%) were found. On day 10, apoptosis remained increased in both hyperglycemic and insulin-treated mice (group 2: 0.44 ± 0.09%; group 5: 0.48 ± 0.08%) compared with normal pancreas (0.04 ± 0.03%; $P < 0.001$). In contrast, on day 30, β-cell apoptosis was increased in grafts exposed to sustained hyperglycemia (group 3: 0.37 ± 0.03%) but not in normoglycemic mice (group 6: 0.12 ± 0.02%); β-cell mass was selectively reduced in islets exposed to hyperglycemia (group 3: 0.046 ± 0.02 mg; group 6: 0.102 ± 0.009 mg; $P < 0.01$). In summary, even in optimal conditions, ~60% of transplanted islet tissue was lost 3 days after syngeneic transplantation, and both apoptosis and necrosis contributed to β-cell death. Increased apoptosis and reduced β-cell mass were also found in islets exposed to chronic hyperglycemia, suggesting that sustained hyperglycemia increased apoptosis in transplanted β-cells.
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ANOVA, analysis of variance; BrdU, 5-bromo-2′deoxyuridine; PBS, phosphate-buffered saline; PLSD; protected least significant difference; STZ, streptozocin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

The amount of transplanted β-cell mass is essential in the outcome of islet transplantation: a critical islet mass, higher than initially predicted, must be transplanted to achieve normoglycemia, and late function and failure depend on the number of initially transplanted islets (1–7). Moreover, the scarcity of islet tissue available for transplantation severely limits the number of transplants that can be performed in diabetic subjects. The recently reported success of human islet transplantation has further emphasized the imbalance between supply and demand of islet tissue for transplantation (8–10). Therefore, preventing the loss of transplanted islets is not only crucial to improving the outcome of the graft but also to reducing the amount of islet tissue required to achieve normoglycemia and to optimize the number of transplants that can be performed with the current limited supply of islets. To define strategies to preserve the transplanted β-cell mass, it is essential to know the extent of β-cell loss and to identify the mechanisms of β-cell destruction and protection.

Transplanted islets are particularly vulnerable in the first days after transplantation (11). Nonspecific inflammation at the grafted site, which may be partly related to damage to islets during isolation (12,13), technical problems during the transplantation process (14), an insufficient amount of transplanted tissue (15), hypoxia of transplanted islets (16), the metabolic condition of the recipient (17), or the absence of survival factors present in the nonendocrine pancreas (18) have been suggested to play a role in the initial fate of transplanted islets. Although increased β-cell apoptosis was recently reported (11), little is known about the events that take place in these initial days after transplantation, and the extent of β-cell loss after transplantation has never been directly quantified.

Several strategies have been reported to offer a protective effect on transplanted islets, but their effect on transplanted β-cell mass has not been quantified, and the mechanism has rarely been investigated (19–21). Using a syngeneic islet transplantation model, we recently showed that insulin treatment improved the outcome of transplanted islets, and definitive normoglycemia was achieved with the transplantation of a β-cell mass that was insufficient to restore normoglycemia in non–insulin-treated mice (17,22). However, the effects of normoglycemia on the crucial initial days after transplantation, and particularly on the preservation of transplanted β-cell mass, were

not examined. On the other hand, we have consistently found that sustained hyperglycemia has a detrimental effect on transplanted islets that have shown limited β-cell replication and a progressive reduction in graft β-cell mass suggestive of increased β-cell death (15,23). However, β-cell death has not been previously investigated in transplanted islets exposed to long-term hyperglycemia. Thus, the aims of this study were to obtain an accurate assessment of the initial fate of transplanted islets by directly quantifying β-cell mass and death, to determine whether transplantation into normoglycemic, insulin-treated, diabetic recipients could reduce the vulnerability of transplanted islets in the initial days after transplantation, and to determine whether β-cell death was increased in transplanted islets exposed to short- and long-term hyperglycemia.

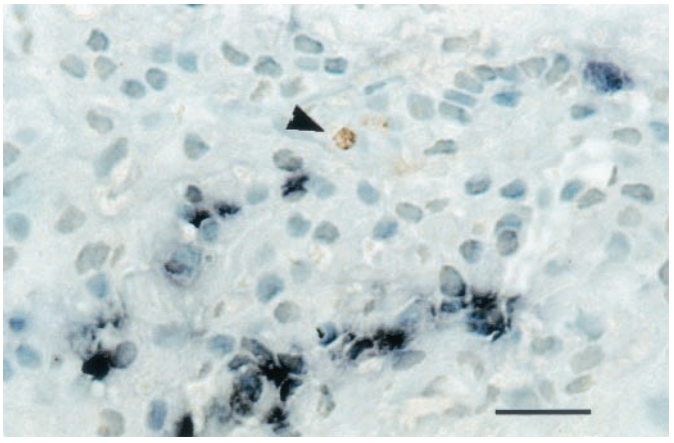

FIG. 1. Micrograph showing an islet graft on day 3 after transplantation to a diabetic insulin-treated mouse (group 4). Section was double stained for TUNEL (immunoperoxidase) and for the endocrine non–β-cells of the islets (alkaline phosphatase) and counterstained with hematoxylin, as described in RESEARCH DESIGN AND METHODS. Arrowhead shows the apoptotic, TUNEL-positive nucleus of a β-cell. Magnification bar = 20 μm.

RESEARCH DESIGN AND METHODS

Male inbred C57BL/6 mice (B&K Universal, Humberside, U.K.), aged 8–12 weeks, were used as donors and recipients of transplantation. The recipients were made diabetic by a single intraperitoneal injection of streptozocin (STZ; Sigma Immunochemicals, St. Louis, MO), 180 mg/kg body wt, freshly dissolved in citrate buffer (pH = 4.5). Before transplantation, diabetes was confirmed by the presence of hyperglycemia, weight loss, and polyuria. Only those mice with a blood glucose >20 mmol/l on a minimum of two consecutive measurements were transplanted. Blood glucose was determined between 9:00 and 11:00 A.M. in nonfasting conditions, unless otherwise stated. Blood was obtained from the snipped tail with a heparinized microcapillary tube, and glucose was measured with a portable glucose meter (Accutrend Sensor; Boehringer Mannheim, Mannheim, Germany). Animals were kept under conventional conditions in climatized rooms with free access to tap water and standard pelleted food.

Experimental groups. STZ-induced diabetic mice were randomly distributed among groups after the transplantation of 100 syngeneic islets, an insufficient β-cell mass to consistently restore normoglycemia in this model (17,23). At 7–14 days after STZ injection, groups 1 (n = 7), 2 (n = 9), and 3 (n = 13) were transplanted, and groups 4 (n = 8), 5 (n = 10), and 6 (n = 7) were started on insulin. Insulin-treated groups were transplanted after 7 days on insulin and a minimum of 2 days of normoglycemia (blood glucose <8.3 mmol/l), and the insulin implants were removed 10 days after transplantation. Insulin was given as two subcutaneous implants of sustained insulin release (LinBits; Linshin Canada, Scarborough, Canada) as previously described (17). The grafts were harvested 3 (groups 1 and 4), 10 (groups 2 and 5), or 30 (groups 3 and 6) days after transplantation.

A control group (n = 6) of normal nontransplanted mice had their blood glucose and body weight determined weekly.

Islet isolation and transplantation. Islets were isolated by collagenase (Collagenase P; Boehringer Mannheim Biochemicals) digestion of the pancreas as previously described (10). Isolated islets were hand-picked under a stereomicroscope two or three times, until a population of pure islets was obtained. Because the size of the islets determines the extent of hypoxia-mediated β-cell destruction (16), only those islets <200 μm in diameter were collected and used for transplantation in an effort to reduce β-cell death after transplantation. Islets were counted into groups of 100 islets and transplanted under the left kidney capsule of the recipient on the day of the isolation. After transplantation, the capsulotomy was cauterized with a disposable low-temperature cautery pen (AB Medica, Barcelona, Spain), and the lumbar incision was sutured.

Graft harvesting. The kidney was exposed, and the kidney capsule surrounding the graft was incised and removed with the graft. Immediately after transplantation, the islets were in loose contact and not fully adhered to the kidney capsule, preventing the consistent harvesting of the whole transplanted tissue; however, on day 3 after transplantation, the grafts were compact enough to ensure a complete recovery of the transplanted tissue. The extraction was performed under the microscope, and after the retrieval of the graft, the kidney cortex was carefully examined to ensure that no islet tissue was left in place. When a complete recovery of the graft was not achieved, the animal was excluded. After removal from the kidney, the graft was immediately immersed in 4% paraformaldehyde–phosphate-buffered saline (PBS), then in 30% sucrose-PBS solution, and then processed for paraffin embedding.

Apoptosis detection with terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method. Two methods were used to determine β-cell apoptosis: the TUNEL technique and propidium staining. Two-micrometer sections were double-stained by immunoperoxidase for apoptotic nuclei using the TUNEL technique (In Situ Cell Death Detection Kit ApopTag; Intergene, Oxford, U.K.) and by alkaline phosphatase for β-cells or for the endocrine non–β-cells of the islets. The TUNEL method used terminal deoxynucleotidyl transferase to catalyze the polimerization of residues of digoxigenin-nucleotide to free 3′OH DNA strand breaks resulting from DNA degradation. The sections were deparaffinized, rehydrated, and incubated with 20 μg/ml proteinase K (Sigma) for 15 min at room temperature. Then, they were labeled according to the manufacturer's instructions. Labeled nuclei were detected using an anti-digoxigenin antibody fragment conjugated with peroxidase, and the peroxidase reaction was visualized with 3,3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide (Fig. 1). Then, sections were washed in double-distilled water and placed in 15% glacial acetic acid solution for 15 min to block the endogenous alkaline phosphatase activity, incubated with PBS plus 5% lamb serum (Gibco BRL), washed with 0.2% PBS-Tween, and subsequently stained with a cocktail of antibodies (Dako, Carpinteria, CA): rabbit anti–swine glucagon (final dilution 1:1,000), rabbit anti–human somatostatin (final dilution 1:1,000), and rabbit anti–human pancreatic polypeptide (final dilution 1:500). We stained the endocrine non–β-cells instead of the β-cells because severe hyperglycemia, expected in non–insulin-treated groups 1, 2, and 3, is associated with β-cell degranulation, resulting in negative or weak staining. Moreover, we previously found a reduction in insulin content during insulin treatment (17) that could also weaken the staining in groups 4 and 5. In addition, the double-staining of nuclei and cytosol could be difficult to discern. To validate the method, additional graft sections from normoglycemic group 6 and from the pancreas of control mice were double-stained with TUNEL and with a guinea pig anti–swine insulin antibody (Dako) (final dilution 1:500), as reported previously for the quantification of β-cell replication (23), and the results were compared with those obtained with the staining of the endocrine non–β-cells. The sections were incubated overnight at 4°C with the insulin antibody or with the cocktail of antibodies, washed with 0.05% PBS-Tween, incubated with a swine anti–rabbit IgG as secondary antibody, and washed with 0.05% PBS-Tween and Tris-chloride (pH = 8.2). Then, they were incubated with a rabbit alkaline phosphatase anti-alkaline phosphatase (APAAP; Sigma), and stained with 5-bromo-4-chloro-3-indolyl phosphatase/nitroblue tetrazolium liquid substrate system (BCIP/NBT; Sigma) for 45 min. Sections were counterstained with Mayer's hematoxylin. β-Cells and apoptotic nuclei were identified and counted using an Olympus BH-2 microscope connected to a video camera with a color monitor. When assessing apoptotic nuclei, we excluded regions with necrosis. β-Cell apoptosis was expressed as percentage of TUNEL-positive β-cells. A minimum of 1,000 cell nuclei were counted per graft, the sections were systematically sampled, all endocrine nuclei were counted, and a second section was included when needed.

β-Cell apoptosis of islets before transplantation was determined in six groups of 100 islets isolated on different days. After isolation, the islets were washed in PBS and pelleted in 4% paraformaldehyde fixative. Sections of the islet pellet were double-stained for TUNEL and for endocrine non–β-cells, and β-cell apoptosis was expressed as percentage of TUNEL-positive β-cells. In addition, β-cell apoptosis in normal pancreas was determined in five pancreas

TABLE 1
Characteristics of experimental groups

Group	n	Insulin	Tx	Day of graft removal	Insulin: Blood glucose (mmol/l)	Insulin: Body weight (g)	Transplantation: Blood glucose (mmol/l)	Transplantation: Body weight (g)	Graft-removal: Blood glucose (mmol/l)	Graft-removal: Body weight (g)
1	7	−	+	3	—	—	32.4 ± 0.6	24.0 ± 0.7	21.9 ± 1.5	24.3 ± 0.8
2	9	−	+	10	—	—	33.3 ± 0.0	23.4 ± 0.6	24.7 ± 2.2	24.5 ± 0.5
3	13	−	+	30	—	—	32.3 ± 0.6	23.2 ± 0.4	24.7 ± 1.7	23.0 ± 0.6
4	8	+	+	3	29.8 ± 1.8	22.9 ± 0.7	4.2 ± 0.5	24.3 ± 0.8	3.7 ± 0.4	24.7 ± 0.7
5	10	+	+	10	31.5 ± 1.2	21.7 ± 0.6	3.3 ± 0.5	25.1 ± 0.4	2.9 ± 0.3	25.4 ± 0.3
6	7	+	+	30	30.7 ± 1.5	22.5 ± 0.5	4.5 ± 0.6	25.1 ± 0.4	7.1 ± 0.7	27.0 ± 0.2
Control	6	−	−	—	6.3 ± 0.1	24.7 ± 0.2	6.5 ± 0.2	26.4 ± 0.2	6.9 ± 0.3	27.4 ± 0.2

Values are means ± SE. In the control group, the columns show body weight and blood glucose values determined in normal non-STZ injected, nontransplanted animals of similar age to STZ-injected and transplanted groups. Tx, islet transplantation.

from control C57BL/6 mice. A midlaparotomy was performed, and the pancreas was exposed, the animal was killed, and the pancreas was immediately excised and fixed in 4% paraformaldehyde. To determine β-cell apoptosis, sections of the pancreases were double-stained and counted as described for grafts and isolated islets.

Apoptosis detection by propidium iodide staining. Graft sections were stained for insulin and propidium iodide as previously described (11,24). Staining with propidim iodide allows the detection of condensed or fragmented nuclei characteristic of apoptotic cells. Sections were stained for the endocrine β-cells using a guinea pig anti–swine insulin antibody (Dako) (final dilution 1:500) for 48 h at 4°C. A swine anti-rabbit IgG was used as secondary antibody. The sections were stained with 3,3′-diaminobenzimide tetrahydrochloride (Sigma Immunochemicals) and hydrogen peroxide (Merck, Darmstadt, Germany). Then, they were washed with distilled water, soaked with PBS (pH 7.4), and incubated for 30 min with propidium iodide (Sigma Immunochemicals) 100 μg/ml and RNAse A (Sigma Immunochemicals) 100 μg/ml at 37°C. Sections were washed twice with PBS and mounted with an aqueous media (Fluoroprep; Dako). The islet tissue was identified on bright field with the insulin staining, and apoptotic cells were identified by the characteristic condensed or fragmented nuclei of cells examined under fluorescent scope with a rhodamine filter. The results were expressed as the percentage of apoptotic over total β-cell nuclei.

β-Cell replication. To determine transplanted β-cell replication, mice were injected with the thymidine analog 5-bromo-2′deoxyuridine (BrdU; Sigma), 100 mg/kg body wt i.p, 6 h before harvesting the graft. Grafts were fixed in 4% paraformaldehyde as described for apoptosis detection. Before incubation with anti-BrdU antibody, slides were pretreated with HCl solution (3 mol/l) for 20 min, washed with borax solution (0.1 mol/l) for 10 min, and incubated with 0.5 mg/ml trypsin for 5 min at room temperature. The sections were double stained with immunoperoxidase for BrdU using a Cell Proliferation Kit (Amersham, Amersham, U.K.) and for the endocrine non–β-cells of the islets using a cocktail of antibodies: anti-glucagon, anti-somatostatin, and pancreatic polypeptide, as previously described in detail (24). After immunoperoxidase staining, β-cells and $BrdU^+$ β-cells were counted using an Olympus BH2 microscope connected to a video camera with a color monitor. β-Cell replication was expressed as percentage of BrdU-positive β-cells, and at least 1,000 cells were counted per graft.

β-Cell replication was measured in the pancreases of five normal C57BL/6 mice that were injected with BrdU 6 h before pancreas excision. Pancreases were fixed and double-stained for BrdU and endocrine non–β-cells as described for grafts, and β-cell replication was expressed as the percentage of BrdU-positive β-cells.

Individual β-cell area. The mean cross-sectional area of individual β-cells, a measure of β-cell size, was determined on the immunoperoxidase-stained sections of grafts and isolated islets with image-analytical software (AnalySIS 3.0; Soft Imaging System, Münster, Germany). The use of this new image analysis system has increased the accuracy of the measurements and reduced the overestimation of β-cell size found with the electronic planimetry program that we previously used (15,17,23). The cross-sectional area of transplanted β-cells was measured in the same sections used for β-cell mass, replication, or apoptosis quantification. The individual β-cell area on the day of transplantation was determined in sections from the six groups of 100 isolated islets used to measure the initially transplanted β-cell mass. For both grafts and isolated islets, the perimeter of the β-cell tissue on a random field was carefully traced on the computer's monitor to exclude any other tissue, and the total β-cell area in that field was measured. Then, the β-cell nuclei in the same field were counted (209 ± 10 nuclei per sample). To calculate the area of the individual β-cells, the total β-cell area in the field was divided by the number of β-cell nuclei.

β-Cell mass. Methods used for measurement of β-cell mass have been described in detail (15,23). β-Cell mass was measured by point-counting morphometry (25) on the same immunoperoxidase-stained sections used to determine β-cell apoptosis, using a 48-point grid to obtain the number of intercepts over β-cells, over endocrine non–β-cells, and over other tissue. β-Cell mass was obtained by multiplying the weight of the graft by the relative β-cell volume.

The β-cell mass of islets at the time of transplantation was determined in six groups of 100 islets isolated on different days. After isolation, the islets were washed in PBS and pelleted in 4% paraformaldehyde fixative, any excess of paraformaldehyde was removed by capillary action, and the pellet was weighed. The β-cell mass was obtained by multiplying the weight of the islets by the percentage of β-cell volume, determined by planimetry on sections of the islet pellets.

Islet cell necrosis. The area of necrosis was measured by point-counting morphometry on the same sections used to quantify β-cell mass. The necrotic area was expressed as the percentage of intercepts over necrotic tissue divided by intercepts over islet tissue (β- and non–β-cells) and necrotic area.

Statistical analysis. Results were expressed as means ± SE. Differences between means were evaluated by one-way analysis of variance (ANOVA). The Fisher's protected least significant difference (PLSD) method was used to determine specific differences between means when determined as significant by ANOVA main effects analysis. $P < 0.05$ was considered significant.

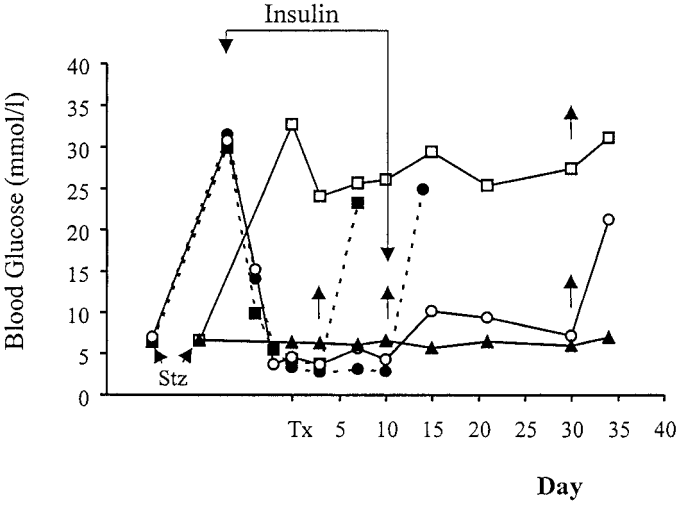

FIG. 2. Evolution of blood glucose in experimental groups. Stz, day of STZ injection; Tx, day of islet transplantation; ↑; graft harvesting. Group numbers correspond to transplanted groups shown in Table 1. □, Groups 1–3; ■, group 4; ●, group 5; ○, group 6; ▲, control group. Values are means ± SE.

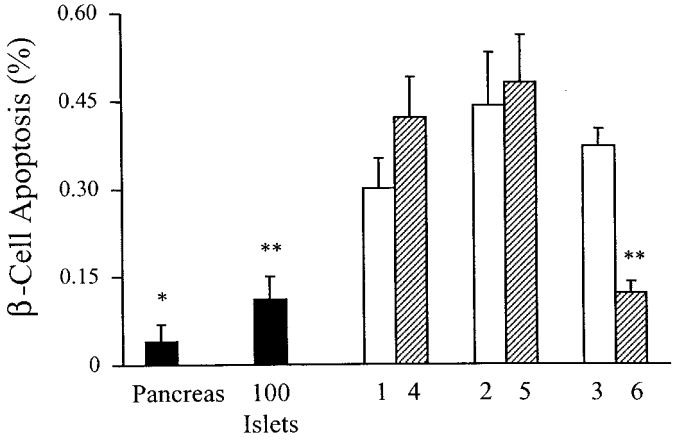

FIG. 3. β-Cell apoptosis in normal pancreas (■), 100 isolated islets (■), and islets transplanted to non–insulin-treated mice (□) and to insulin-treated mice (▨). Group numbers on the *x*-axis correspond to transplanted groups shown in Table 1. Values are means ± SE. ANOVA, $P < 0.001$; Fisher PLSD, *$P < 0.005$ for pancreas vs. groups 1–5; **$P < 0.05$ for 100 isolated islets vs. groups 2–5 and 6.

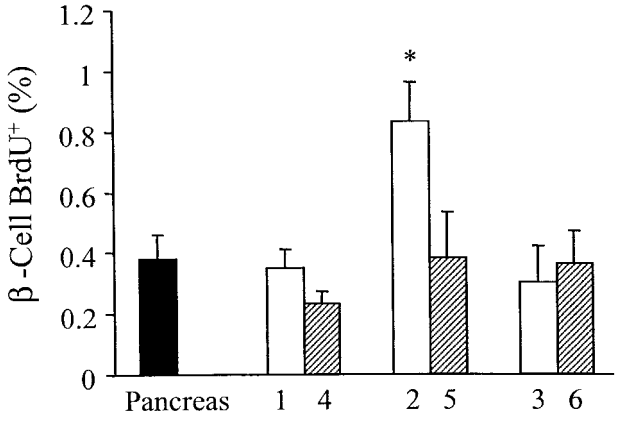

FIG. 4. β-Cell replication in normal pancreas (■) and in islets transplanted to non–insulin-treated mice (□) and to insulin-treated mice (▨). Group numbers on the *x*-axis correspond to transplanted groups shown in Table 1. Values are means ± SE. ANOVA, $P < 0.001$; Fisher PLSD, *$P < 0.001$ for group 2 vs. all other groups.

RESULTS

Metabolic evolution. The characteristics of experimental groups are summarized in Table 1, and the evolution of blood glucose is shown in Fig. 2. All groups were comparable in blood glucose and body weight when injected with STZ as well as when islets were transplanted (groups 1–3) or when insulin implants were placed (groups 4–6). As expected, the noninsulin-treated groups remained severely hyperglycemic after transplantation, and most (70%) insulin-treated mice maintained normoglycemia when insulin was withdrawn. Only those mice that achieved strict normoglycemia after insulin withdrawal (70%) were included in the study. In the non–insulin-treated groups, one mouse achieved normoglycemia after transplantation and was excluded from the study. In the insulin-treated groups, blood glucose was lower than in the control group during insulin treatment (insulin-treated group: 3.70 ± 0.18 mmol/l; control group: 6.30 ± 0.09 mmol/l; $P < 0.001$). When insulin was discontinued, blood glucose was slightly and transitorily increased on day 15 (group 6: 10.1 ± 1.7 mmol/l; control group: 5.65 ± 0.13 mmol/l; $P = 0.035$) and day 21 after transplantation (group 6: 9.31 ± 1.13 mmol/l; control group: 6.46 ± 0.31 mmol/l; $P = 0.045$), but normoglycemia had been achieved on day 30, when the grafts were harvested (Table 1). Severe hyperglycemia (>20 mmol/l) recurred in all normoglycemic mice when the graft was harvested.

Graft morphology. Graft morphology was similar in the hyperglycemic and insulin-treated groups on day 3. After transplantation, the islets tended to fuse, as has been previously reported (11), and on day 3, areas of necrosis were evident, generally in the center of the endocrine tissue in both the normoglycemic and hyperglycemic groups. The location of the areas of necrosis in the center of the endocrine tissue and the exclusive presence of endocrine tissue at the transplantation site at this early time after engraftment indicated that the necrotic areas corresponded to islet tissue. The area of necrosis was 26.8% in insulin-treated mice and 30.7% in hyperglycemic mice. On day 10, no necrosis was visible in the grafts, but areas containing erythrocytes ("erythrocyte lakes") were now identified. Although these areas were larger and more abundant in hyperglycemic mice, with the endocrine tissue often distributed as a rim of surrounding cells, they were also identified in insulin-treated mice. On day 30 after transplantation, grafts in normoglycemic mice showed a homogeneous, compact, and healthy endocrine tissue, as has been previously described in successful islet transplants. In contrast, in hyperglycemic mice, the endocrine tissue was disrupted, showing important fibrosis and the persistence of large erythrocyte lakes.

β-Cell apoptosis by TUNEL. β-Cell apoptosis was similar when β-cells were directly visualized using an insulin antibody and when the endocrine non–β-cells of the islets were stained with the cocktail of antibodies, both in control pancreas (insulin staining: 0.03 ± 0.01%; cocktail staining: 0.04 ± 0.03%) and transplanted β-cells of normoglycemic group 6 (insulin staining: 0.14 ± 0.02%; cocktail staining: 0.12 ± 0.02%), indicating the validity of staining the endocrine non–β-cells to determine β-cell apoptosis.

β-Cell apoptosis was not significantly increased immediately after islet isolation (0.11 ± 0.04%) compared with control pancreases. On days 3 and 10 after transplantation, β-cell apoptosis was significantly increased in both hyperglycemic mice (group 1: 0.30 ± 0.05%; group 2: 0.44 ± 0.09%) and normoglycemic, insulin-treated mice (group 4: 0.42 ± 0.07%; group 5: 0.48 ± 0.08%). In contrast, on day 30 after transplantation, β-cell apoptosis remained increased only in grafts exposed to sustained hyperglycemia (group 3: 0.37 ± 0.03%), whereas grafts from normoglycemic mice showed a β-cell apoptosis similar to that of control pancreas (group 6: 0.12 ± 0.02%) and significantly lower than the other transplanted groups (Fig. 3).

β-Cell apoptosis by propidium iodide. β-Cell apoptosis was quantified with propidium iodide in 3–5 grafts from each group. The results showed strong parallelism with those of the TUNEL method and confirmed that β-cell apoptosis was similarly increased on days 3 (group 1: 0.41 ± 0.14%; group 4: 0.33 ± 0.12%) and 10 (group 2: 0.30 ± 0.11%; group 5: 0.40 ± 0.09%). As found with the TUNEL staining, on day 30 after transplantation, β-cell apoptosis was increased in hyperglycemic mice (group 3: 0.26 ± 0.01%) but not in normoglycemic animals (group 6: 0.09 ± 0.06%).

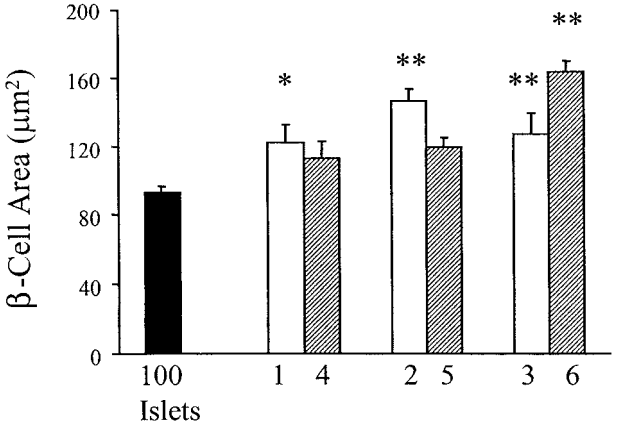

FIG. 5. Area of individual β-cells in 100 isolated islets (■) and in islets transplanted to non–insulin-treated mice (□) and to insulin-treated mice (▨). Group numbers on the *x*-axis correspond to transplanted groups shown in Table 1. Values are means ± SE. ANOVA, $P < 0.001$; Fisher PLSD, $*P < 0.05$ for 100 isolated islets vs. group 1; Fisher PLSD, $**P < 0.01$ for 100 isolated islets vs. groups 2, 3, and 6.

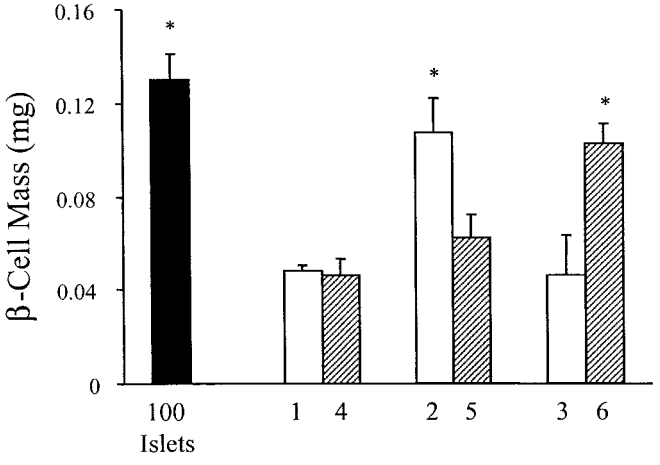

FIG. 6. β-Cell mass in 100 isolated islets (■) and in grafts transplanted to non–insulin-treated mice (□) and to insulin-treated mice (▨). Isolated islets show the initially transplanted β-cell mass. Group numbers on the *x*-axis correspond to transplanted groups shown in Table 1. Values are means ± SE. ANOVA, $P < 0.001$; Fisher PLSD, $*P < 0.001$ for 100 isolated islets and groups 2 and 6 vs. all other groups.

β-Cell replication. β-Cell replication was increased in transplanted islets exposed to 10 days of hyperglycemia (group 2: 0.83 ± 0.13%) compared with β-cell replication in normal pancreas (0.38 ± 0.08%) and the other transplanted groups (Fig. 4).

β-Cell area. The individual cross-sectional area of β-cells in isolated islets was 93 ± 3 μm^2. On day 3 after transplantation, transplanted β-cell size was similar in hyperglycemic and normoglycemic, insulin-treated mice, although it was mildly increased in hyperglycemic mice (group 1: 122 ± 10 μm^2) compared with initially transplanted islets. In contrast, 10 days after transplantation, β-cell size was clearly increased in hyperglycemic mice (group 2: 146 ± 7 μm^2) and remained increased on day 30 after transplantation (group 3: 127 ± 12 μm^2). After insulin withdrawal, β-cell size increased in normoglycemic mice (group 6: 163 ± 6 μm^2) (Fig. 5).

β-Cell mass. The initially transplanted β-cell mass was 0.13 ± 0.011 mg. This value is lower than our previously reported β-cell mass in groups of 100 islets (17,21), reflecting the smaller size of islets selected for transplantation in the current experiments, as described in RESEARCH DESIGN AND METHODS. β-Cell mass was dramatically and similarly reduced on day 3 after transplantation in hyperglycemic and insulin-treated mice (group 1: 0.048 ± 0.002 mg; group 4: 0.046 ± 0.007 mg) compared with the initially transplanted β-cell mass. In hyperglycemic mice, β-cell mass increased transitorily on day 10 after transplantation (group 2: 0.107 ± 0.015 mg) but was reduced again after 30 days of exposure to hyperglycemia (group 3: 0.046 ± 0.017 mg). In contrast, in insulin-treated mice, β-cell mass remained reduced on day 10 (group 5: 0.062 ± 0.010 mg), but it increased to a value similar to the initially transplanted β-cell mass when metabolic demand was increased after insulin withdrawal (group 6: 0.107 ± 0.015 mg) (Fig. 6).

DISCUSSION

In this study, we have shown that even when transplanted in optimal conditions, more than half of the islet tissue was lost in the initial days after syngeneic transplantation, and that both apoptosis and necrosis contributed to this early β-cell death. Although transplantation into normoglycemic, insulin-treated recipients improved the long-term outcome of the graft, the initial β-cell death and mass loss were similar in islets transplanted to normoglycemic and hyperglycemic recipients. Thus, insulin treatment did not improve the initial preservation of transplanted β-cell mass in the initial days after transplantation. In contrast, increased apoptosis and reduced β-cell mass were found in islets exposed to long-term hyperglycemia but not in normoglycemic mice, suggesting that sustained hyperglycemia increased β-cell death in transplanted islets.

This is the first study to directly quantify the extent of β-cell loss initially after transplantation, a period considered crucial in the outcome of the graft. The vulnerability of transplanted islets in the immediate posttransplantation period has been recently reported (11), but β-cell mass was not quantified. We have found that on day 3 after transplantation, 30% of the engrafted tissue was necrotic, and β-cell apoptosis was 10 times higher than in the normal pancreas. Overall, β-cell apoptosis and necrosis resulted in the loss of 60% of the initially transplanted β-cell mass. This extensive islet damage is even more impressive considering that the transplants were performed in optimal conditions: a syngeneic model was used to exclude rejection and avoid the toxic effects of immunosuppressive drugs on β-cells (26,27), the islets were selected by size to minimize the deleterious effect of hypoxia (16), and they were transplanted into an optimal site (28).

Increased apoptosis and necrosis resulted in the dramatic β-cell mass reduction found on day 3 after transplantation, but their respective contributions to total β-cell death could not be precisely quantified. On one hand, the extent of islet necrosis may be even higher than that measured because some absorption of the dead islet tissue may have already started on day 3. On the other hand, the duration of β-cell apoptosis is unknown; therefore, it is not possible to calculate the rate of β-cell apoptosis per day. However, the 10-times increased β-cell apoptosis indicates that it played a significant role in total β-cell death, even

moreso considering that apoptosis is a fairly rapid process (29). The reasons for the discrepancy with the higher apoptotic figures found in a similar model (11) are not evident. We used two methods to determine β-cell apoptosis that yielded concordant results, and β-cell apoptosis in our control pancreases was similar to that described by most groups. Nevertheless, similar to our data, Davalli et al. (11) reported that apoptosis in transplanted islets was also 10 times higher than in normal pancreases.

β-Cell death in the initial days after transplantation was similarly increased in islets transplanted to insulin-treated diabetic recipients and to hyperglycemic recipients. In previous studies, we have reported an improved outcome of islets transplanted to normoglycemic, insulin-treated diabetic recipients (17,22,30); these islets showed better β-cell function and preserved insulin content compared with islets transplanted to hyperglycemic recipients (17,30). It could be expected that hyperglycemia would increase the death of transplanted islets because it increases oxygen consumption of islets (16) and may delay the revascularization of islet grafts (31,32). However, β-cell apoptosis, necrosis, and β-cell mass loss were similar in islets transplanted to hyperglycemic and to insulin-treated recipients, suggesting that the initial β-cell destruction after transplantation is not dependent on the metabolic conditions of the recipients.

On day 10 after transplantation, β-cell mass increased in grafts transplanted to hyperglycemic recipients but not in those transplanted to insulin-treated mice. β-Cell apoptosis was increased in both groups, and no necrosis was found in either group, indicating that the differences in β-cell mass resulted from the β-cell hypertrophy and hyperplasia of islets exposed to 10 days of hyperglycemia. These results are consistent with the increased β-cell proliferation and size reported in the initial days of exposure to hyperglycemia (23,33,34). Although the negative effects of chronic hyperglycemia, confirmed by our data on day 30 after transplantation, have been repeatedly reported, the recovery of β-cell mass on day 10 after transplantation suggests that a shorter exposure to hyperglycemia could have a beneficial effect on transplanted islet mass. On the other hand, the increased β-cell apoptosis detected on day 10 in islets transplanted to insulin-treated mice may have been appropriate because these islets were not needed to maintain normoglycemia and could even put the recipients at risk for hypoglycemia. The existence of an apoptotic pathway to downregulate β-cell mass in vivo has been reported in postpartum rats (35) and in animals bearing transplantable insulinomas (36). The moderate hypoglycemia found in some insulin-treated mice may have also contributed to increased β-cell apoptosis in this group.

On day 30, β-cell apoptosis was similar in islets transplanted to normoglycemic mice and in the pancreas of control animals, indicating that increased apoptosis is not a characteristic of transplanted islets per se—a concern that had been previously raised (11). The normal β-cell apoptosis and increased β-cell size found on day 30 after transplantation, along with the transitory hyperplasia that probably took place in the initial days after insulin withdrawal (17,23), resulted in the expansion of transplanted β-cell mass in normoglycemic mice. In contrast, β-cell apoptosis remained increased in islets exposed to chronic hyperglycemia. This apoptosis could not be offset by the limited β-cell replication of islets exposed to long-term hyperglycemia (15,17,23) and the inability to further increase β-cell size, and resulted in the reduction of β-cell mass. Overall, the data support the hypothesis that β-cell mass declined in transplanted islets exposed to chronic hyperglycemia because of increased β-cell death. Korsgren et al. (37) had also suggested that prolonged exposure to a hyperglycemic environment could lead to β-cell death, at least in genetically susceptible islets, and recently, glucose-induced apoptosis was demonstrated both in vivo and in vitro in the diabetes-prone *Psammomys obesus* (38).

To our knowledge, we have provided the first evidence that chronic exposure to severe hyperglycemia can result in increased apoptotic β-cell death in animals that are not genetically predisposed to diabetes. Formation of advanced glycation end products (39), increased production of reactive oxygen species through glycation reaction (40) and, recently, protein modification by the *O*-linked monosaccharide N-acetylglucosamine (*O*-Glc-Nac) (41) have been suggested as possible mediators of chronic hyperglycemia-induced β-cell damage. In addition, the severe reduction in insulin content (17) and decreased insulin gene transcription (30) in transplanted islets exposed to chronic hyperglycemia could deprive the β-cells from the suggested survival role of insulin. In models with genetic susceptibility to type 2 diabetes or in islets with increased sensibility to the toxic effects of hyperglycemia, such as human islets (42,43), chronic exposure to less severe hyperglycemia could be sufficient to increase β-cell apoptosis, leading to β-cell mass reduction and contributing to the progression of type 2 diabetes.

The insufficient amount of islet tissue available for transplantation is a major obstacle for islet transplantation and is further aggravated by the high number of islets required for successful transplantation. The substantial loss of islet tissue that we have identified in the initial days after transplantation due to increased β-cell apoptosis and necrosis may contribute to this elevated islet requirement. Understanding the mechanisms involved in transplanted β-cell death and survival is essential to develop therapeutic strategies aimed to protect transplanted islets.

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