Preservation of Human Islet Cell Functional Mass by Anti-Oxidative Action of a Novel SOD Mimic Compound

Rita Bottino,[1] A.N. Balamurugan,[1,2] Suzanne Bertera,[1] Massimo Pietropaolo,[1] Massimo Trucco,[1] and Jon D. Piganelli[1]

The most commonly used technical approach to isolate human pancreatic islets intended for allotransplants generates a product that is hampered by mechanical and chemical insults, which dramatically reduce the mass of viable and functional transplantable cells. We tested a novel class of antioxidant chemical compounds (SOD mimics: AEOL10113 and AEOL10150) to protect human islets from oxidative stress in order to improve the preservation of the isolated tissue. Addition of SOD mimic in culture, after isolation, allowed for the survival of a significantly higher islet cell mass. Functional behavior and phenotypic cell characteristics of the SOD-treated islet preparations were preserved, as was the capacity to normalize diabetic mice, even when a marginal mass of islets was transplanted. The addition of SOD mimic during isolation, before culture, further reduced early cell loss. These results indicate that prompt interventions aimed at blocking oxidative stress can improve human islet survival, preserving a functional islet mass two- to threefold larger than the one usually obtained without adding any antioxidant compound. The ability to preserve functional islets without a dramatic loss represents a major advantage considering the scarce availability of islet tissue for clinical transplantation. *Diabetes* 51:2561–2567, 2002

In children, the whole pancreas transplant cannot be used, due to technical negative consequences, whereas transplantation of pancreatic islets has long been considered a potential curative treatment for type 1 diabetes (1,2). Unfortunately, transplanted islets are susceptible to allogeneic rejection and recurrence of autoimmunity, which impose the use of a powerful protective immunosuppressive regimen (2). Damage associated with nonspecific inflammatory events occurs not only in the microenvironment in which the islet grafts find their final location after clinical transplantation, i.e., in the liver sinusoids, but also during the separation of the islets from the surrounding tissue matrix (3–5). Human islet isolation is achieved by a combination of mechanical and enzymatic digestive processes, followed by separation of the islets from the exocrine cells by density gradients (6). The isolation technique itself influences yield and functional properties of the final islet preparation (7). Variables such as donor and tissue characteristics, ischemia time, and endotoxic contamination can additionally contribute to render islet cells more susceptible to the injury triggered during isolation (8,9). Their effects combined with nonspecific inflammatory events can play a fatal role for days and weeks after isolation, leading to the high rate of primary graft failure seen in islet cell transplantation (10). This might also explain why each recipient of islet transplants must receive islet tissue from more than one donor to obtain an adequate islet β-cell mass for achieving euglycemia (2). In culture, a consistent reduction of the islet mass has been documented (11), and it appears to occur despite the identification of improved culture conditions (12,13).

Isolation insults result in osmotic, mechanic, ischemic, and oxidative stress for islets (7). These stressful events may increase the production of either proinflammatory cytokines or free radicals by passenger leukocytes and by the islet cells themselves, leading ultimately to β-cell dysfunction and death (14,15). Studies have shown that the destruction of the extracellular matrix (4) during isolation leads to islet apoptosis. Furthermore, islet cell death may also occur by necrosis (16). Cells that die by necrosis release immunogenic intracellular proteases into the microenvironment. These proteases have the ability to activate macrophages to produce proinflammatory cytokines and generate additional free radicals (17). It is conceivable to postulate that early blockage of nonspecific inflammation, proinflammatory cytokine production, and free radicals generation would prevent islet cell loss, improving islet engraftment and survival. It has recently been reported that upregulation of the ubiquitous free radical–scavenging stress protein Heme oxygenase-1 protected both rodent islets and β-cell lines from apoptosis and improved their in vivo function (18). Furthermore, it is known that the expression of the free radical–scavenging enzyme manganese superoxide dismutase (MnSOD) in insulinoma cells prevents interleukin (IL)-1β–induced cytotoxicity and reduces nitric oxide production (19). It has also been reported that the addition of protease inhibitors during islet isolation yields higher islet functional mass (20). Prevention of oxidative stress by means of more powerful compounds may further improve the survival of isolated human islets in culture and in vivo.

From the [1]Department of Pediatrics, Division of Immunogenetics, Diabetes Institute, University of Pittsburgh, School of Medicine, Rangos Research Center, Children's Hospital of Pittsburgh, Pittsburgh, Pennsylvania; and the [2]Department of Surgery, Thomas E. Starlz Transplantation Institute, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania.

Address correspondence and reprint requests to Jon D. Piganelli, PhD, Division of Immunogenetics, Diabetes Institute, Rangos Research Center, Children's Hospital of Pittsburgh, 3460 Fifth Ave., Pittsburgh, Pennsylvania 15213. E-mail: jdp51+@pitt.edu.

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ELISA, enzyme-linked immunosorbent assay; KRBB, Krebs-Ringer bicarbonate buffer.

TABLE 1
Donor characteristics

	Human pancreas (HP)	Age (years)	Cold ischemia time (min)	Sex	BMI (kg/m^2)	% islets/whole preparation
Study group 1	48	68	7	M	22.9	60–70
	49	50	15	F	30.2	60–70
	50	54	5	M	27.7	65–75
	52	49	12	M	30.4	65–75
	53	51	8	F	17.6	60–70
	54	46	6	F	17.6	70–80
	55	17	8	F	24.2	70–80
	57	65	7	F	23.4	60–65
	58	55	7	M	22.4	60–70
Study group 2	60	24	11	M	31	60–65
	68	48	9	F	37	60–65
	71	57	10	M	29	30–35

Two synthetic metalloporphyrin-based SOD mimics (AEOL10113 and AEOL10150), designed with a redox-active metal center that catalyzes the dismutation of $O_2^{\cdot -}$ in a manner similar to the active metal sites of the mammalian Cu-, Zn-, or Mn- containing SODs (21–23), were used in this study. The SOD mimics AEOL10113 and 10150 have a broad antioxidant specificity, which includes scavenging of $O_2^{\cdot -}$, H_2O_2, $ONOO^-$, $NO^{\cdot}$, and lipid peroxyl radicals (24,25). The protective effects of SOD mimics on several tissues and cell types, such as endothelium and neurons, have been reported (26–28). Recently we demonstrated that SOD mimic protects a β-cell line from the effect of diabetogenic agents and prevents or delays the onset of diabetes in young NOD.*scid* recipients after adoptive transfer of a diabetogenic T-cell clone (29). The ability of the SOD mimics to efficiently scavenge free radicals and to protect cells from oxidative stress and apoptosis warrants their use for the preservation of isolated human islets.

The aim of this study was to assess whether the addition of the SOD mimics AEOL10113 or AEOL10150 could protect human islet cells from free radical induced–damage during isolation and after culture. In the initial experiments, SOD mimics were used as a culture supplement after islet isolation, which resulted in an improved preservation of islet cell mass. From the initial experiments, we surmised that the addition of the SOD mimics earlier, during the isolation phase, may provide even greater protection from free radical–induced damage. To address this additional hypothesis, SOD mimics were added already to the solution containing Liberase used for tissue digestion. To avoid differences in donor and organ variables, we utilized the same pancreatic gland as experimental and control tissues. The results obtained demonstrate that the introduction of the SOD mimic during isolation leads to a significantly higher (70 vs. 40%) islet cell mass than the one obtained with no treatment. SOD mimic treated islets are physiologically functional with respect to insulin secretory response in vitro and to their capacity to restore normoglycemia in immunodeficient diabetic recipient mice.

RESEARCH DESIGN AND METHODS

Human islets. Human pancreata were obtained from CORE (Center for Organ Recovery and Education, Pittsburgh, PA) and from NDRI (National Disease Research Interchange, Philadelphia, PA). Twelve pancreata failing the standard criteria for the use of whole pancreas or islet transplantation were processed (Table 1). To more comprehensively test our compound, we set up to utilize all available organs without applying any exclusion criteria. The cold ischemia time was 9 ± 2 h ranging from 5 to 15 h. The age was 49 ± 4 years (range 17–68 years) and BMI was 26 ± 2 kg/m^2 (data are mean ± SE). The final islet yield (IEQ/g) for the pancreatic preparations (n = 9) obtained with the semiautomated method, which consisted of purity between 60 and 80%, was 4,628 ± 749 IEQ/g (range 1,790–7,631, median 4,542).

The percentage of islets over whole preparation was determined immediately after isolation, by dithizone (Sigma, St. Louis, MO) staining (30). This was 60–80% in the first group of nine islet preparations and 60, 60, and 30%, respectively, in the last three preparations. The yield obtained using the second series of experiments (stationary digestion) was 1,744 ± 676 IEQ/g.

Culturing of purified human islets in the presence of SOD mimic. The initial series of experiments involved the addition of SOD mimic after islet isolation, as a culture supplement. Metalloporphyrin SOD mimics AEOL 130 and AEOL 150 were provided by Incara Pharmaceuticals. The islets were isolated using the method described by Ricordi et al. (6) with minor modifications. Before purification, the digest was incubated in cold University Wisconsin solution for 45 min. The islet-enriched fractions were purified using discontinuous Euro-ficoll density gradients and processed in a COBE 2991 Cell Separator (Gambro, Lakewood, CO). The islets were cultured for 7 days (37°C, 5% CO_2) in CMRL-1066 culture medium supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 2 mmol/l L-glutamine (Life Technologies, Grand Island, NY) with or without SOD mimic at the final concentration of 34 μmol/l. Fresh culture medium (with or without SOD mimic) was replaced three times per week and islet samples were taken for assessment of DNA content and for functional studies at selected time points.

Determination of islet cell mass and viability. DNA content has been used as an indirect measure of cell mass, since the clustered nature of the islets, together with the nonendocrine contaminants, makes direct counting inappropriate (31). DNA content was measured in samples of the islet preparations from both control and SOD-treated groups, using a Pico Green dsDNA Quantitation Kit following the manufacturer's protocol (Molecular Probes, Eugene, OR). Islet viability was determined by simultaneous staining of live and dead cells using a two-color fluorescence assay (Calcein-AM and Propidium Iodide, Molecular Probes) (32). The percentage of viable and dead cells was estimated in both control and SOD-treated groups.

Static glucose challenge and insulin content measurement. Handpicked islets from control and SOD-treated groups were subjected to static glucose challenge in Krebs-Ringer bicarbonate buffer (KRBB) (pH 7.35) containing 10 mmol/l HEPES and 0.5% BSA (Sigma). After conditioning, the islets were incubated in KRBB containing low (2.8 mmol/l) and high (20 mmol/l) glucose concentrations for 1 h. At the end of the glucose challenge, insulin extraction was carried out to determine islet insulin content (33). The insulin levels were measured by enzyme-linked immunosorbent assay (ELISA) (ALPCO, Windham, NH).

Immunocytochemistry. Samples of the purified islet preparations were fixed in Bouin's solution for 1 h and then transferred to 10% buffered formalin. Using standard procedures, the islet samples were stained for immunoreactive proinsulin, glucagon, CK19 (Scytek Laboratories, Logan, UT), and amylase

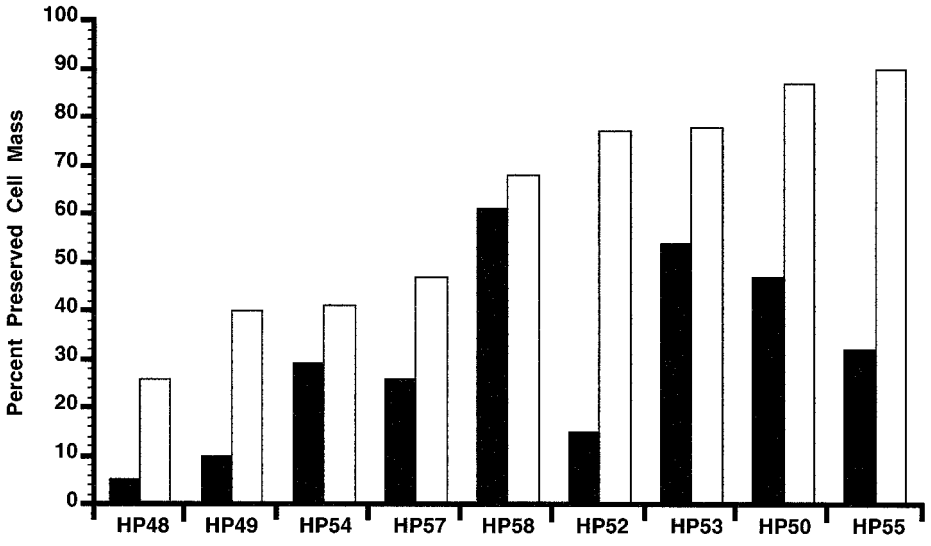


FIG. 1. Percentage of preserved islet cell mass between days 2 and 7 during culture in control (■) and SOD mimic (□)-treated groups (n = 9). DNA content measured at day 2 (24 h after isolation) has been arbitrarily considered as the starting reference value. Differences between groups were statistically significant (P = 0.02, by Mann-Whitney U test).

(Biogenex, San Ramon, CA), and the percentage of positive cells was counted in both control and SOD-treated groups.

Perifusion protocol of islet insulin release. Whether addition of SOD mimic to the islet medium induces unregulated insulin release was determined by a perifusion protocol in three different human islet preparations, carried out under constant, physiologic glucose concentration. Groups of 100 hand-picked islets (diameter 100–150 μm) cultured under control conditions for 4–7 days were perifused with KRBB containing 5.6 mmol/l glucose. During the 90-min perifusion period, the SOD mimic was added between 30 and 60 min. The insulin concentration of the elution samples was measured by ELISA (ALPCO).

Administration of SOD mimic during islet isolation. In the second series of experiments, SOD mimic was administered during the isolation phase as well as during culture. The pancreatic lobe was divided into two parts (body and tail) of similar size (27 ± 3 g) that were randomly assigned to control and experimental conditions. During isolation, SOD mimic (concentration 34 μmol/l) was delivered to the half of the pancreatic tissue together with Liberase, by intraductal injection. In the control group, only Liberase was infused. The split organs are not amenable to automatic processing; therefore, stationary digestion was carried out in parallel on the two organ segments. The samples were digested for 30 min at 37°C with periodic shaking. Cell dissociation was finalized by manual teasing of the tissue. Cells and aggregates were filtered through a 500-μm mesh and collected in cold RPMI-1640 culture medium (Life Technologies) containing 10% heat-inactivated FCS. Before COBE processing, the pancreatic digests were incubated with cold UW solution with or without SOD mimic (34 μmol/l) for 45 min. Purification and culture were then continued as described in the first series of experiments.

Islet transplantation under the kidney capsule of diabetic mice. Islet grafts of 200–1,000 IEQ (islet equivalents) from four different organs were cultured in the presence of SOD mimic or kept in control conditions for at least 2 h before transplantation. Streptozotocin-induced (250 mg/kg body wt; Sigma) diabetic NOD-*scid* or Rag 1 mice (The Jackson Laboratories, Bar Harbor, ME) exhibiting nonfasting blood glucose levels of >300 mg/dl were used as recipients. Animals were anesthetized by intraperitoneal injection of Avertin (0.30–0.40 mg/g body wt) in accordance with the guidelines of National Institutes of Health and the Institutional Animal Research Care Committee of the University of Pittsburgh. Control or SOD-treated islets were transplanted under the mouse kidney capsule (34). Successful engraftment was defined by reduction of glycemic levels to <200 mg/dl after transplantation. After 4–7 weeks post-transplantation, normalized recipients underwent nephrectomy to remove the islet graft. Return to hyperglycemia was interpreted as indirect proof of islet graft function.

Statistical methods. Statistical analysis was carried out by Student's t test, Mann-Whitney U nonparametric test, and Kaplan and Meier analysis. P values <0.05 were deemed statistically significant.

RESULTS

Presence of SOD mimic preserves islet cell mass. Isolated islets from nine donor pancreata were divided into two groups and cultured in either CMRL-1066 alone or supplemented with SOD mimic. The results in Fig. 1 demonstrate that in all experimental groups (n = 9), a threefold mean increase in cell mass was observed when compared with controls (P = 0.02). It should be noted that the difference in DNA content between control and experimental groups was appreciable starting from day 2 (24 h after isolation) and steadily observed over the remaining culture period. However, in the first 24 h of culture, a similar decrease in cell mass was observed in control and SOD-treated islet preparations (data not shown), accounting for 20–40% of the initial cell mass. These data demonstrate that addition of SOD mimic reduces cell loss significantly over time, although it appears that there is no protective effect on those cells that die very early, within 24 h after isolation.

Cell viability. Double-fluorescence viability was performed to determine whether DNA content might be affected by the presence of dead nondegraded cells in higher proportion within the SOD mimic–treated preparations. The results indicate that in both control and SOD-treated aggregates, a similar number of viable and dead cells was present; however, the total islet number was higher in SOD-treated than in the control group. At a later stage of the culture period, control preparations showed higher dead cell contents, but the difference did not reach statistical significance (Table 2).

Static glucose challenge and in vitro islet function. Islet glucose responsiveness was assessed by a static glucose challenge method between days 3 and 5 after isolation (Table 3). Upon glucose stimulation, insulin release, expressed as absolute value or as a percentage of the insulin content, and stimulation indexes were similar,

TABLE 2
Islet functional viability

	Culture days 1–4		Culture days 5–10	
Groups	Viable %	Dead %	Viable %	Dead %
Control	80 ± 5	20 ± 5	92 ± 5	8 ± 5
SOD mimic	79 ± 5	21 ± 5	86 ± 5	14 ± 5

Data are means ± SE. Percentage of viable and dead cells present in islet preparations (n = 9). Differences are not statistically significant.

TABLE 3
Islet functional glucose responsiveness

Groups	Stimulated insulin secretion (μU insulin · islet^{-1} · h^{-1})	% Insulin content	Stimulation index
Control	13 ± 2	3 ± 1	5.5 ± 1.5
SOD mimic	14 ± 2	3 ± 0.5	5.8 ± 1

Data are means ± SE of five islet preparations. Stimulated insulin secretion values are obtained by subtracting basal from 20 mmol/l glucose-induced insulin release.

regardless of the culture treatment. Also, basal insulin release did not differ between groups (data not shown).

We also studied the effect of addition of SOD mimic on insulin release during a 90-min perifusion protocol of isolated human islets (n = 3) under constant glucose concentration. The results demonstrate that SOD mimic supplementation does not affect insulin secretion (data not shown). Basal release was similar in all groups, regardless of the addition of SOD mimic to the medium.

Stimulated insulin secretion values are obtained by subtracting basal from 20 mmol/l glucose-induced insulin release. Values are mean ± SE of five islet preparations.

Characterization of the total cell mass. To exclude the possibility that higher DNA contents in SOD-treated preparations could be attributed to selective survival of nonislet tissue, we characterized the islet preparations (n = 5) by immunocytochemistry. The relative proportion of proinsulin (β-cells), glucagon (α-cells), amylase (exocrine), and CK19 (ductal cells) was determined (Fig. 2*A*). In all cases, cell composition was minimally different between SOD mimic–treated and control groups. Both groups showed a reduction in amylase immunoreactive cells that changed from values as large as 20% at start to negligible values after culture, regardless of the culture treatment ($P < 0.01$). When the proportion of proinsulin-positive cells (% of β-cells) was expressed as a fraction of DNA content, used as an indicator of cell number, the results illustrate that SOD mimic treatment accounts for a 1.3- to a 4.5-fold increase in β-cell mass (Fig. 2*B*). These data demonstrate that treatment of islets with SOD increases overall β-cell mass without appreciable dysregulation in islet function.

Early administration of SOD mimic reduces cell loss. The aim of the three additional experiments was to determine whether administration of SOD mimic during the digestion might play a more protective role on cell survival than its addition in culture after isolation. To avoid the impact of donor variables on the quality of the isolated islets, we have adopted the technical approach of splitting the organs in two parts. After infusion of Liberase solution with or without SOD mimic, an arbitrary incubation time of 30 min was maintained in all groups. Importantly, the presence of SOD mimic did not appear to affect the digestion duration. Treated and control preparations for each donor organ yielded islet fractions of similar

A

Donor Tissue Exp. Groups	Pro-Insulin	Glucagon	Amylase	CK19	Time of Evaluation
HP49	43	15	25*	20	at start
Control	50	21	5	14	after culture
SOD	55	11	5	12	
HP50	43	15	20*	10	at start
Control	40	12	9	12	after culture
SOD	46	17	3	12	
HP53	45	6	25*	15	at start
Control	50	5	<5	12	after culture
SOD	65	5	5	20	
HP54	55	10	10*	20	at start
Control	35	15	0	20	after culture
SOD	40	17	0	22	
HP55	35	12	30*	18	at start
Control	47	25	0	22	after culture
SOD	46	24	0	20	

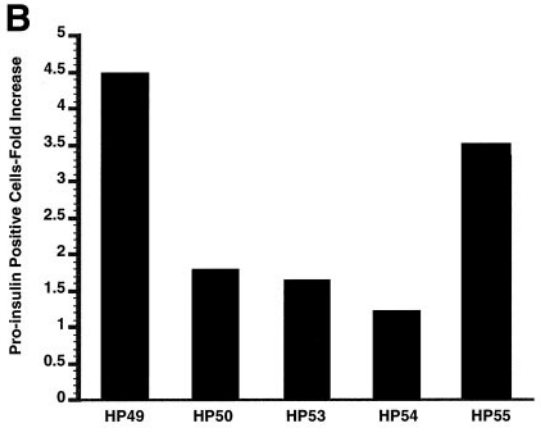


FIG. 2. *A*: Immunocytochemical characterization of islet preparations from five human pancreata before and after culture. The number of positive cells was counted and expressed as percentage of the total. The percentage of proinsulin-positive cells was maintained over the culture period in all preparations irrespective of the treatment. After culture, a statistically significant (*$P < 0.01$, by Student's t test) decrease in amylase positive cells was observed in both control and SOD mimic–treated preparations. *B*: Bar graphs show the fold-increase in preserved β-cell mass in SOD mimic–treated preparations compared with control over the culture time. β-cell mass was calculated by combining DNA content as an indicator of cell number with immunocytochemical analysis (35).

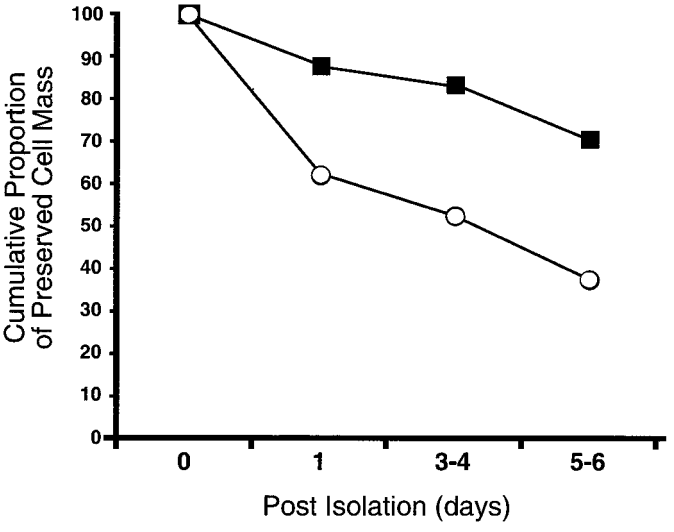
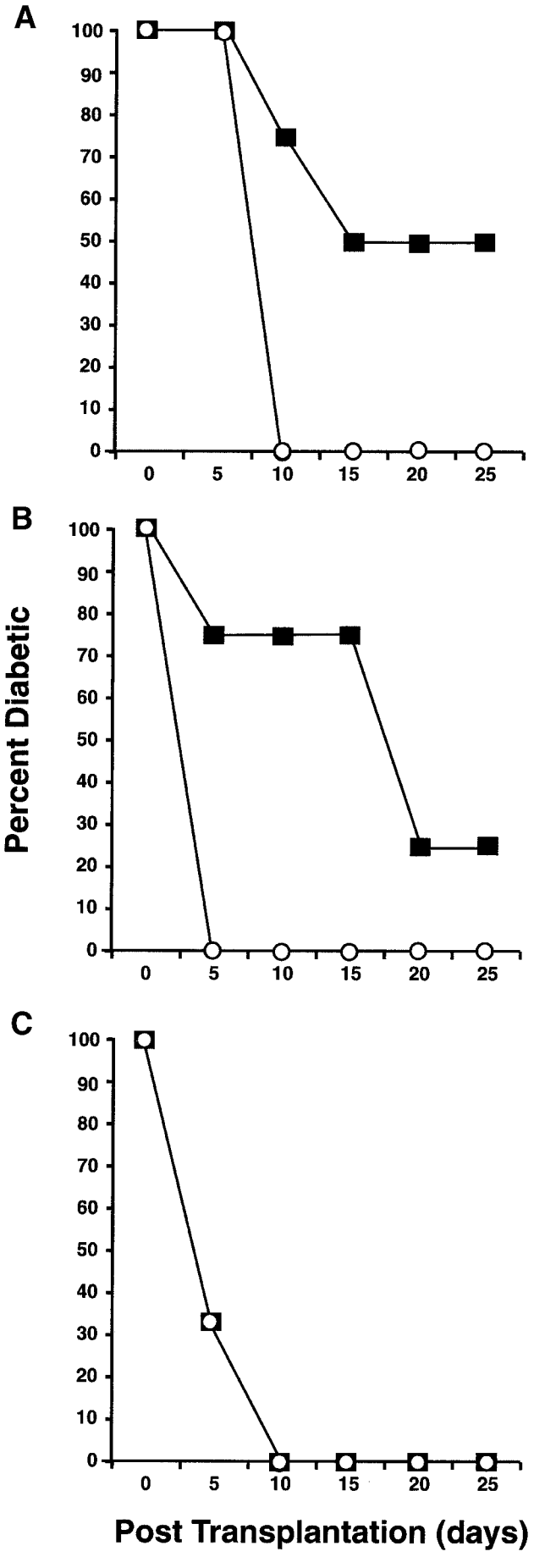


FIG. 3. Survival rate for islet preparations of the SOD mimic group (■) and the control group (○). The donor pancreata (n = 3) were divided in two fractions and treated with or without SOD mimic during isolation. From the Kaplan and Meier analysis, it can be inferred that islet cell loss reduced in SOD-treated pancreatic tissue as compared with control tissue (log rank, P = 0.0001). Remarkably this difference was seen after 24 h.



purity. Figure 3 demonstrates the relative variations in DNA content of control and experimental islet preparations after isolation. The data indicate that in all three cases, a significant ($P = 0.0001$) increase in islet cell mass was appreciable 24 h after isolation in the SOD-treated organs and that the difference was maintained over time. Treated and control islets were tested for viability and functional capacity in vitro. In both cases >80% of viable cells were counted among the islet preparation samples, with no difference associated with the treatment. The values of insulin content and basal and stimulated insulin release per islet were also not different between the two groups (data not shown). This demonstrates that the addition of the SOD mimic during digestion resulted in greater numbers of functional islets with respect to controls.

Normalization of diabetic mice. Diabetic recipient mice were randomly assigned to receive islet transplants from control and SOD mimic–treated groups (Fig. 4). The first three recipients of each group received an islet mass of 700–1,000 IEQ. All six recipients normalized the glucose levels within the first week of the post-transplant period. When the number of transplanted islets was reduced to <400 IEQ per graft, all recipients ($n = 9$) of SOD-treated islets normalized, while in the control group, three recipihpents of islet grafts (composed of 200, 220, and 400 IEQ, respectively) never corrected glucose levels. In all successful transplants, animals maintained normoglycemia for >30 days. In the cases in which the graft was removed by nephrectomy, all mice returned to a hyperglycemic state.

DISCUSSION

It has been shown that islet cells can be maintained in culture for variable periods of time after isolation. This is achieved at the cost of some cell loss and functional impairment (11). Donor tissue characteristics (e.g., medical history, age of donor, cause of death, and length of hospitalization) (36) and morphological conditions of the pancreas are some of the possible causes for cell loss, but a major role seems to be played by the degree of stress and relative cell damage triggered during the isolation procedure (7). In order to bypass problems related to cell loss during culture, most clinical trials of islet transplantation use preferentially freshly isolated islets (2). Although this approach warrants higher β-cell content, it is not valuable to predict the fate of the islet graft and its metabolic performance. The possibility to maintain islet cells in culture for short periods of time, without remarkable loss, offers the potential advantage of performing quality control tests and, in view of transplants, to operate a more appropriate selection of the recipients (11).

The mechanisms behind islet cell loss after isolation are partially understood. Studies have shown that islet cells are sensitive to the effect of extracellular matrix destruction that occurs during isolation and leads to apoptosis (4). An additional contribution to cell death is likely due to metabolic and mechanical damage that occurs before

FIG. 4. Metabolic capacity of islet grafts in vivo. Glycemia normalization time after islet transplantation of variable islet mass. *A*: Islet grafts 200–220 IEQ; control $n = 4$, SOD mimic $n = 5$. *B*: Islet grafts ~400 IEQ; control $n = 4$, SOD mimic $n = 4$. *C*: Islet grafts 700–1,000 IEQ; control $n = 3$, SOD mimic $n = 3$. ■, control; ○, SOD mimic.

isolation, related to hours of ischemia and storage in cold preservation fluid. These factors might additionally contribute to aggravate the noxious effects of the isolation procedure on the tissue. It has already been proven that by targeting oxidative stress, islet cell damage can be partially prevented (18).

In this study, we investigated the ability of SOD mimic to prevent islet cell loss in culture at physiologic temperature. Addition of SOD mimic to the culture medium after isolation did not affect the cell loss that occurs during the first 24 h, but it significantly reduced it in the following culture days. These results demonstrate that loss of islet cells during culture can be reduced, allowing for better characterization of islet function before transplant. The "preserved cell mass" was composed of proinsulin as well as other pancreatic cell types; exocrine cells died during culture, irrespective of the treatment.

The observation that SOD mimic plays a role in improving cell survival in culture has further led to a second working hypothesis: by exposing pancreatic tissue to SOD mimic at an earlier stage during isolation (already at the time of enzyme delivery), cell loss could be further reduced. To exclude the effect of donor variables on the yield and function of the final preparations, we utilized the same donor tissue, isolated and cultured the islet-enriched fractions, and examined the difference in islet cell mass over time. As hypothesized, substantially higher islet cell mass was preserved in SOD-exposed preparations. These data support the concept that early oxidative events contribute to cell loss, and consequent blockage of these mechanisms results in higher cell survival. In vitro glucose responsiveness and in vivo capacity of the survived cells have further shown that SOD mimic does not impair islet functional performance. Moreover, it appears to enhance islet graft capacity in vivo. The higher degree of success promoted in obtaining stable euglycemia in the mice transplanted with SOD-treated islets was more evident when a marginal islet mass was used. Meaningful correlation with improved metabolic parameters of islet function or secretory capacity determined in vitro are difficult to make. Whether the beneficial effect of SOD mimic directly targets islet cells performance or protects the transplant microenvironment from inflammatory events, potentially harmful to graft survival, is not yet clear and needs further investigation. If we also consider that SOD mimic protects islet β-cells from the autoimmune attack of a diabetogenic T-cell clone in vivo (29), then the two effects can be compounded. The absence of apparent toxicity of this compound allows its use during islet isolation and its possible administration in vivo after transplantation.

The progressive improvement of cell survival observed by anticipating tissue exposure to SOD mimic indicates that further attempts at reducing oxidative and free radical stress at the stage of organ harvesting might be worthwhile. It might be advantageous to suggest adding SOD mimic to the procurement solution during organ perfusion to obtain more successful protective effect.

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