Mitochondrial Targeting with Antioxidant Peptide SS-31 Prevents Mitochondrial Depolarization, Reduces Islet Cell Apoptosis, Increases Islet Cell Yield, and Improves Posttransplantation Function

Dolca A. Thomas,*† Craig Stauffer,* Kesheng Zhao,‡ Hua Yang,* Vijay K. Sharma,* Hazel H. Szeto,‡ and Manikkam Suthanthiran*†

Departments of *Medicine, †Transplantation Medicine, and ‡Pharmacology, Weill Medical College of Cornell University, New York-Presbyterian Hospital-Weill Cornell, New York, New York

Apoptotic cell death is a defined pathway for islet cell demise, and mitochondrial dysfunction contributes to islet cell apoptosis. The hypothesis that the novel peptide D-Arg-2', 6'-dimethyltyrosine-Lys-Phe-NH₂ (SS-31), previously shown to target inner mitochondrial membrane and prevent oxidative damage of neuronal cells and other cell types, optimizes pancreatic islet isolation and improves posttransplantation function in recipients with diabetes was investigated. Herein is demonstrated that SS-31 readily penetrates intact mouse islets, preserves mitochondrial polarization, reduces islet cell apoptosis, and increases islet cell yield. Optimization of islet isolation is demonstrable after SS-31 pretreatment of islet (pancreas) donor mice and with the addition of SS-31 to reagents that are used in the isolation of mouse islets. The supplementation of in vitro culture medium with SS-31 reduced islet cell apoptosis and increased the viability of human islets, as ascertained by dual-parameter flow cytometry analysis. In a stringent marginal islet cell mass transplantation model (200 mouse islets transplanted under the renal capsule of syngeneic diabetic mice) and using islets that were derived from old mice (>24 wk), SS-31 treatment was associated with prompt and sustained normoglycemia, whereas the untreated islet graft recipients remained diabetic. Our data suggest a novel strategy to optimize islet isolation and reduce the need for multiple pancreata to achieve insulin independence in the recipient with type 1 diabetes. Because SS-31 was effective with “extended” islet donors, it is hypothesized that the antioxidant SS-31 may serve to increase the pool of eligible organ donors.


Existing barriers to islet cell transplantation include the need for islets from multiple pancreata to achieve insulin independence and the progressive decline in islet function over the years (1,2). Successful islet isolation remains a challenge as well and is influenced by factors such as organ donor age, body mass index, and cold ischemia time (3). Organ donors who are older than 50 yr currently account for 35% of all deceased donors, and only 6% of pancreata are retrieved for transplantation (4), in part because islets from old donors exhibit impaired glucose responsiveness (5). The inability to use the old donors worsens further the existing disparity between pancreas supply and demand.

Stressors that are present during pancreas procurement, preservation, and isolation and after transplantation contribute to islet cell demise, and ongoing research efforts are directed at the development of strategies to islet cell apoptosis (6). Early events that precede cellular dysfunction and demise include disruption of mitochondrial membrane potential, mitochondrial permeability transition pore (MPT) formation, and increased intracellular accumulation of reactive oxygen species (ROS) (7,8). Mutations in mitochondrial DNA accumulate with aging, and the mutations promote apoptosis (9). A direct link among aging, mitochondrial mutations, and ROS accumulation is far from fully established. Excess ROS production, however, results in mitochondrial impairment, uncoupling of oxidative phosphorylation, and the release of proapoptotic cytochrome c from the mitochondrial membrane and cellular demise (10).

Antioxidants such as coenzyme Q, vitamin E, and N-acetylcysteine have been investigated to reduce oxidant injury and limit cellular damage (11–13). These compounds have wide extracellular and intracellular distribution, fail to accumulate within mitochondria, and require conjugation with lipophilic cations for mitochondrial targeting (14,15). Their ability to enter mitochondria and accumulate within the matrix depends on the inner mitochondrial membrane potential and its gradient, a requirement that may limit their capacity to permeate depolarized cells. Moreover, the accumulation of these antioxidant cations within the mitochondrial matrix can lead to dissipation of inner mitochondrial membrane potential, an event that often leads to cellular demise. As a result, these agents exhibit narrow therapeutic ranges.
Genetic strategies are being explored to bolster antioxidant defense. *Ex vivo* transfer of manganese superoxide dismutase gene to mouse islets has extended islet graft function in autoimmune diabetic mice (16). It has been observed that the islets from mice that overexpress glutathione peroxidase and the two isoforms of superoxide dismutase but not the islets from single- or double-transgenic mice improve blood glucose control in a marginal islet mass model (17). Recently, it was reported that the expression of X-linked inhibitor of apoptosis protein in human islets (18) or mouse islets (19) reduces islet cell apoptosis and improves islet graft function.

Intraductal administration of l-glutamine has been reported to reduce islet oxidative injury, as assessed by malondialdehyde concentration, and increased glutathione levels, diminished apoptosis, and improved posttransplantation function in diabetic nude mice (20). We investigated whether d-Arg-2,6-diarylhydroxy-L-lysine (SS-31), a novel antioxidant peptide, penetrates intact islets, preserves mitochondrial polarization, reduces islet cell apoptosis, increases islet cell yield, and improves posttransplantation function. We studied SS-31 in vivo of its ability to inhibit, with minimal cellular toxicity, mitochondrial swelling, oxidative cell death, and ischemia-reperfusion injury of cardiac or neuronal cells (21,22). SS-31 has several advantages, including being a small water-soluble peptide, the ability to target and accumulate at the inner mitochondrial membrane in a potential-independent manner, and the ability to reduce islet oxidative injury, as assessed by malondialdehyde concentration, and increased glutathione levels, diminished apoptosis, and improved posttransplantation function in diabetic nude mice (20). We investigated whether d-Arg-2,6-diarylhydroxy-L-lysine (SS-31), a novel antioxidant peptide, penetrates intact islets, preserves mitochondrial polarization, reduces islet cell apoptosis, increases islet cell yield, and improves posttransplantation function. We studied SS-31 in vivo of its ability to inhibit, with minimal cellular toxicity, mitochondrial swelling, oxidative cell death, and ischemia-reperfusion injury of cardiac or neuronal cells (21,22). SS-31 has several advantages, including being a small water-soluble peptide, the ability to target and accumulate at the inner mitochondrial membrane in a potential-independent manner, and the ability to protect against mitochondrial depolarization. We hypothesized that the bioactivities of SS-31 would confer islet cells a survival benefit and optimize islet isolation and transplantation.

**Materials and Methods**

**Chemical and Reagents**

SS-31 was prepared by solid-phase synthesis and provided by Dr. Peter W. Schiller (Clinical Research Institute of Montreal, Montreal, QU, Canada) (23). [3H]SS-31 was prepared by Dr. Peter Schiller and Dr. Geza Toth (Institute of Isotopes, Budapest, Hungary) as described previously (24).

**Mice**

All studies on mice complied with the Institutional Animal Care and Use Committee regulations of the Weill Cornell Medical College. Male DBA/2 (H-2d) mice $>$24 wk of age were used as islet donors, and 8- to 12-wk-old diabetic DBA/2 mice were used as islet graft recipients. Recipient mice were rendered diabetic via a single intraperitoneal injection of streptozotocin (Sigma, St. Louis, MO) at 175 mg/kg. Diabetes was defined as two consecutive blood glucose levels $>$350 mg/dl.

**SS-31 Peptide Treatment**

DBA/2 islet donor mice were treated with SS-31 (3 mg/kg, subcutaneously) twice a day 24 h before and on the day of pancreas harvest for islet isolation. After islet transplantation, the recipient, diabetic DBA/2 mice were treated with SS-31 (3 mg/kg, subcutaneously) twice daily for 10 d.

**Islet Cell Isolation and Transplantation**

Islet isolation was performed as described previously (25). The common bile duct was cannulated, and pancreas was distended by intraductal administration of collagenase at 1 mg/ml. The pancreas was removed and incubated at 37°C for 16 min, and islets were isolated by Ficoll density centrifugation. A total of 1 nM SS-31 was added to all reagents that were used for islet isolation in the SS-31 treatment group. Marginal mass islet cell transplantation was performed by transplantation of 200 islets under the right kidney capsule of diabetic DBA/2 mice. Human islets were isolated from diseased organ donors with a modified Ricordi procedure (26).

**Measurement of Islet Cell Uptake of SS-31**

Intact islet cells were incubated at 37°C for 1 h in RPMI medium that contained 1 nM [3H]SS-31, and radioactivity was measured in the cell lysate and in the medium as described previously (22). The radioactive counts in the medium were subtracted from radioactive counts in the cell lysate, and the counts were normalized for protein content.

**Assessment of Mitochondrial Membrane Potential**

Mitochondrial membrane potential of islet cells was assessed using the fluorescence probe Mitotracker TMRE (tetramethylrhodamine methyl ester; Molecular Probes, Portland, OR). Islet cells were incubated with TMRE for 30 min at 37°C, and fluorescence intensity, at excitation/emission wavelengths of 552/570 nm, was examined using a confocal laser scanning microscope (22).

**Determination of Islet Cell Apoptosis by Flow Cytometry**

Islet cells were dissociated into single cells by incubation with trypsin-EDTA for 7 min. The dissociated islet cells were incubated with propidium iodide (PI) or TO-PRO-3 and annexin V-FITC (annexin V) for 15 min at room temperature. Samples were analyzed on FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) using 488 nm of excitation and a 530/30-nm filter for FITC detection and a 650-nm filter for PI detection. Dual-parameter flow cytometry was used to determine early apoptotic cells (annexin V single-positive cells), late apoptotic/necrotic cells (annexin V/PI or TO-PRO-3 double-positive cells), necrotic cells (PI or T-PRO-3 single-positive cells), or viable cells (annexin V/PI or TO-PRO-3 double-negative cells), as described previously (27).

**Statistical Analyses**

Data are means ± SEM unless otherwise specified as. Two-tailed $P$ value was calculated using the paired *t* test or the independent *t* test for continuous variables and the $\chi^2$ test for dichotomized variables.

**Results**

**Islet Cell Uptake of Antioxidant Peptide SS-31**

An earlier study found cellular uptake of SS-31 by a neuronal cell line N2A (22). Islets, however, are tightly adherent cell clusters, and entry of peptides/proteins may be impaired given its complex cytoarchitecture. [3H]SS-31 was prepared, and we examined whether the antioxidant peptide SS-31 enters intact mouse islets. Figure 1A illustrates that SS-31 readily penetrates intact mouse islets. In four consecutive experiments, the mean (%) SEM of [3H]SS-31 uptake was 70.2 ± 10.3 pmol/mg protein.

**SS-31 Prevents Mitochondrial Depolarization**

Mitochondrial depolarization and the release of cytochrome c into the cytoplasm are critical antecedent events to cell death. TMRE, a fluorescence cationic indicator, is taken up into mitochondria in a potential-dependent manner, and the opening of the mitochondrial permeability transition pore and depolarization leads to diminished labeling of the mitochondrial by...
the fluoroprobe. We used Mitotracker TMRM to determine whether SS-31 preserves mitochondrial polarization.

DBA/2 mice were pretreated with SS-31 or vehicle control 24 h before pancreas harvest for islet isolation. SS-31 also was added to the islet isolation medium that was used to isolate islets from the pancreas that was harvested from the SS-31-pretreated pancreas/islet donors. After isolation of islets from the SS-31-treated or the control mice, the intact mouse islets were incubated with TMRM, and fluorescence was examined using a confocal laser scanning microscope.

Figure 1B shows fluorescence and phase images of islets from vehicle (control) or SS-31–treated mice. It is evident that the uptake of TMRM and the resultant fluorescence intensity of islets from SS-31–treated mice are greater compared with the uptake and fluorescence intensity observed with islets from the control mice (Figure 1B, iii versus i).

**Figure 1.** SS-31 peptide penetrates islet cells, co-localizes within the mitochondria, and preserves islet mitochondrial membrane potential. (A) Islet cell uptake of SS-31. DBA/2 islet cells were incubated with 1 nM of tritium-labeled SS-31 and 1 uM unlabeled SS-31 at 37°C for 1 h. After incubation, radioactivity was measured in the medium and in the cell lysate and the radioactive counts in the medium were subtracted from radioactive counts in the cell lysate and normalized to protein content. In four consecutive experiments, the mean (±SEM) [H3]SS-31 uptake was 70.2 ± 10.3 pmol/mg protein. (B) SS-31 preserves islet mitochondrial potential. DBA/2 mice were pretreated with SS-31 (3 mg/kg, subcutaneously twice daily) or vehicle control 24 h before pancreas harvest for islet isolation. SS-31–pretreated group had 1 nM SS-31 added to the islet isolation reagents. After isolation, tetramethylrhodamine methyl ester (TMRM) uptake was evaluated using confocal laser scanning microscopy. Fluorescence (i) and phase (ii) images of TMRM uptake in the islets from the control mice demonstrate reduced fluorescence uptake, indicating mitochondrial depolarization. In sharp contrast, fluorescence (iii) and phase (iv) images of TMRM uptake in the islets from the SS-31–treated mice demonstrate increased uptake and preserved mitochondrial potential, indicative of SS-31 protective effect.

**Optimization of Islet Isolation with SS-31**

We investigated whether SS-31 optimizes islet isolation and results in increased islet yield. Pretreatment of islet donor mice with SS-31 and supplementation of islet isolation reagents resulted in a significantly higher islet cell yield compared with untreated mice, and the results from nine paired islet isolation experiments that were composed of 34 pancreata in the control group and 32 pancreata in SS-31 treatment group are shown in Figure 2A. The mean (±SEM) islet yield from the pancreata that were harvested from SS-31–pretreated mice was 338 ± 46 islets per pancreas compared with 281 ± 42 islets per pancreas that was retrieved from the control mice (P = 0.001, paired t test).

We determined whether the addition of SS-31 to the islet isolation reagents alone is sufficient to increase islet yield. Results from five paired islet isolations that were composed of 19 pancreata in the control group and 19 pancreata in the SS-31 group demonstrating that the addition of SS-31 to islet isolation reagents (without islet donor pretreatment) results in a significant increase in islet cell yield are illustrated in Figure 2B. The addition of SS-31 to the islet isolation reagents alone increased the islet yield from 241 ± 21 to 312 ± 20 (P = 0.03).

**SS-31 Reduces Islet Cell Apoptosis**

SS-31 treatment, in addition to enhancing islet yield, resulted in a significant decrease in islet cell apoptosis. Dual-parameter flow cytometry analyses of islet cells that stained simultaneously with annexin V and PI demonstrated that the treatment of islet donor mice with SS-31 and supplementation of islet isolation reagents reduced islet cell apoptosis and increased viability. Figure 3, A through D, are individual and mean (±SEM) percentages from five paired islet isolations that were composed of 20 pancreata in the control group and 21 pancreata in SS-31 treatment group. The mean (±SEM) percentage of early apoptotic cells (annexin V–positive cells) was 11.4 ± 2.4% with islets from the control mice and 5.5 ± 1.0% with islets from mice that were pretreated with SS-31 (P = 0.028; Figure 3A). SS-31 treatment reduced late apoptosis/necrosis (annexin V/PI double-positive cells) from 22.7 ± 4.7 to 12.6 ± 1.8% (P = 0.048; Figure 3B) and increased islet cell viability (annexin V/PI double-negative cells) from 47 ± 5.1 to 62 ± 3.5% (P = 0.037; Figure 3D). SS-31 pretreatment of islet donor mice and the addition of SS-31 to the islet isolation reagents, however, did not reduce the percentage of necrotic cells (PI-positive cells): 20 ± 3.2 versus 19 ± 4.7% (P = 0.80; Figure 3C).
SS-31 Improves Posttransplantation Islet Graft Function

We investigated with the use of a marginal islet mass transplantation model whether SS-31 pretreatment–associated decrease in islet cell apoptosis and enhanced viability had a beneficial functional consequence in vivo. The marginal mass islet cell transplantation was performed by transplanting 200 syngeneic islets under the right kidney capsule of diabetic DBA/2 mice. In the marginal islet mass transplantation model, zero of eight recipients of islets that were isolated from pancreas that was harvested from the control mice had successful reversal of hyperglycemia (defined as three consecutive blood glucose levels <200 mg/dl), whereas sustained normoglycemia occurred in five of 10 recipients of islets that were isolated from pancreas from the SS-31–treated donor mice (Figure 4). It is worth noting that the reversal of hyperglycemia was prompt (by day 1 after transplantation) and that the discontinuation of SS-31 treatment of the islet graft recipient on day 10 did not result in return of hyperglycemia.

Effect of SS-31 on Human Islets

Human islets often are cultured in vitro before transplantation, and the in vitro culture protocol offers an opportunity to optimize islet transplant outcome. We determined whether the addition of SS-31 to the in vitro culture medium has an impact on human islet cell apoptosis and viability. Human islets that were isolated from three deceased donors and cultured in vitro for 24 to 72 h at 22 or 37°C in medium that was supplemented without or with SS-31 were examined at the single-cell level. Dual-parameter flow cytometric analyses of human islets that stained with annexin V and TO-PRO-3 are shown in Figure 5; Table 1 is a summary showing that SS-31 supplementation of in vitro culture medium results in a decrease in islet cell apoptosis and in a modest increase in viability of human islets from pancreas donors aged 65 and 45 but not aged 28.

Discussion

The new findings from our study are that the novel antioxidant peptide SS-31 readily penetrates intact islets, prevents mitochondrial depolarization, enhances islet cell yield, reduces apoptosis, and improves posttransplantation function. SS-31 is islet protective when added to the islet isolation reagents alone or when the in vitro culture medium is supplemented with SS-31. Our study also supports the hypothesis that the reduction in islet cell apoptosis and increase in viability may be observed not only with mouse islets but also with human islets.

The islet isolation procedure disrupts islet cells from their extracellular matrix via an enzymatic and mechanical process and subjects the islets to cytokine, oxidative, and ischemic stress (28–30). Increased islet cell damage as a result of excess endogenous protease activity and cellular ATP depletion has been implicated in reduced islet cell recovery after isolation (31,32). Mitochondria are the major source of ATP and intracellular ROS and control cell death (8,33). ROS and reactive nitrogen species can cause damage to all cellular macromolecules, including nucleic acids, proteins, carbohydrates, and lipids. Mitochondria are particularly vulnerable to oxidative damage because they are exposed constantly to high levels of ROS. In addition to mitochondrial lipid peroxidation, protein oxidation and nitrification results in altered function of many metabolic enzymes in the mitochondrial matrix as well as those that comprise the electron transport chain. Although impairment of mitochondrial function may lead to ATP depletion and necrotic cell death, oxidative damage to mitochondria also can result in the release of cytochrome c into the cytosol, which can trigger apoptosis (33). Cytochrome c normally is bound to the inner mitochondrial membrane by association with cardiolipin. Peroxidation of cardiolipin leads to dissociation of cytochrome c and its release through the outer mitochondrial membrane into...
the cytosol. Release of this solubilized pool of cytochrome c may occur by permeabilization of the outer mitochondrial membrane, by oligomerization of Bcl-2 family proteins, or via mitochondrial permeability transition with resultant mitochondrial swelling and rupture of the outer membrane. Cytochrome c in the cytoplasm triggers the caspase cascade by activation of caspase-9.

SS-31 belongs to a family of small cell-permeable peptides that target and concentrate in the inner mitochondrial membrane, the site of ROS generation (21). These peptides can scavenge ROS and reduce mitochondrial ROS production and thereby prevent mitochondrial permeability transition and cytochrome c release (21). SS-31 has been shown to prevent apoptosis that is induced by a lipid hydroperoxide in neuronal cell lines (22) as well as reduce infarct size in a rodent model of myocardial ischemia-reperfusion (34). We therefore investigated the hypothesis that SS-31 is islet protective and optimizes islet isolation and improves posttransplantation function.

SS peptides have been shown to penetrate a variety of cell types, including neuronal cells, intestinal and kidney epithelial cells, and endothelial cells, in a concentration-dependent and temperature-independent manner (35). Because no specialized uptake systems are required for the cellular uptake of SS peptides, it was predicted that SS-31 will be taken up by pancreatic islet cells. Indeed, we found that the uptake by pancreatic islets (70.2 ± 10.3 pmol/mg protein) was even higher than the uptake by neuronal SH-SY5Y cells (approximately 15 pmol/mg protein) (22). With respect to cellular location of SS peptides, confocal laser scanning microscopic studies of intestinal epithelial cells, neuronal cells, and HeLa cells demonstrated localization to the mitochondrion (21). Such mitochondrial targeting also was observed by uptake studies using liver and brain mitochondria (21,23). Therefore, it seems that mitochondria targeting of these SS peptides can be generalized to most, if not all, cell types.

Some additional features of SS peptide family members also
are worthy of note. Unlike other mitochondria-targeted compounds (e.g., MitoQ, MitoE), mitochondrial uptake of SS peptides is not driven by mitochondrial potential, and SS peptides can be taken up even by depolarized mitochondria (21). Second, because the SS peptides are positively charged, they are not delivered into the mitochondrial matrix and their uptake is not self-limiting and they do not cause mitochondrial depolarization even at high concentrations (36).

In our study, pretreatment of islet donor mice and/or the addition of SS-31 to the islet isolation reagents alone resulted in a significant increase in islet cell yield. The increase in islet cell yield may be due to optimized cellular ATP levels and preserved intracellular calcium homeostasis. The intriguing possibility that the antioxidant SS-31 reduces endogenous and exogenous protease activity and optimizes islet isolation remains to be investigated.

A variety of protocols have been used to investigate islet cell demise via the apoptotic and/or necrotic pathway. A wide range, for example from 1 to 24%, has been reported for islet cell apoptosis (28,30,37). Clearly, multiple factors, such as the islet source and type of collagenase used in islet isolation, contributed to the broad range observed, and it is likely that the differential sensitivity of methods that are used for the evaluation of islet cell apoptosis also is a factor.

Our analysis, at the single-cell level of islets that stained simultaneously with annexin V and PI or TO-PRO-3, identified four subpopulations: Single positive for annexin V, double positive for annexin V and PI/TO-PRO-3, single positive for PI/TO-PRO-3, or double negative for annexin V and PI/TO-PRO-3. We classified the annexin V single-positive cells as early apoptotic cells and the double-positive cells as late apoptotic/necrotic cells because it has been reported that late apoptotic cells may become permeable to dyes such as PI (27) and because an apoptotic pathway may convert to a necrotic pathway with progressive energy depletion and impaired caspase activity (38). The PI/TO-PRO-3 single-positive cells, in our study, were classified as necrotic cells. This subpopulation, which was found consistently in our study of islet cell apoptosis and observed as well in the study of Berney et al. (39), is found in some but not in all studies of apoptosis/necrosis of lymphoid cells or tumor cells (40,41).

We found that SS-31, besides increasing islet cell yield, is effective in reducing islet cell apoptosis and increasing their viability. The cytoprotective benefits were observed, albeit to a lesser extent, with the addition of SS-31 to the islet isolation medium alone and also with human islets that were incubated in vitro in culture medium that was supplemented with SS-31. The beneficial effects that were observed by the addition of SS-31 to the islet isolation medium alone is important because it can obviate the need for donor pretreatment before organ retrieval. However, additional studies are needed to determine whether the addition of SS-31 to the islet isolation medium alone results in improved posttransplantation function.

ROS promotes opening of the MPT pore that results in dissipation of the mitochondrial potential, uncoupling of oxidative phosphorylation, and rupture of the outer mitochondrial membrane (42). The rupture of the outer mitochondrial membrane allows the release of cytochrome c into the cytosolic compartment, where it induces activation of the caspase cascade that is responsible for apoptotic cell death (43). By targeting the inner mitochondrial membrane, SS-31 can scavenge ROS at their site of origin and prevent ROS-mediated MPT and cytochrome c release and ultimately prevent apoptosis (21). At the concentration used (1 nM), SS-31...
Table 1. In vitro culture of human islets with SS-31

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Table 1. In vitro culture of human islets with SS-31

aHuman pancreata were removed en bloc from deceased organ donors (DD) and were preserved using the two-layer (University of Wisconsin solution/perfluorodecalin) organ preservation system. The cold ischemia time (CIT; the period between pancreas removal and the beginning of islet isolation) for individual pancreas is shown. The islets were isolated from the DD pancreata using the modified Ricordi procedure and were incubated in culture medium that was supplemented with SS-31 for 24 to 72 h and at 22 or 37°C. At the end of in vitro culture, the islets were dispersed and stained with annexin V and propidium iodide (PI) or TO-PRO-3 iodide. Dual-parameter flow cytometry analysis was used to discriminate early apoptotic cells (annexin V single-positive cells), late apoptotic/necrotic cells (annexin V and PI or TO-PRO-3 single-positive cells), necrotic cells (PI or TO-PRO-3 double-positive cells), or viable cells (annexin V/TO-PRO-3 double-negative cells) are shown. The vertical and horizontal lines in D, E, and F are the cursor settings that were used to determine the percentage of cells in each quadrant.
cannot scavenge extracellular ROS or cytoplasmic ROS because in vitro studies have shown that >1 uM is necessary for ROS scavenging in vitro. However, because SS-31 concentrates approximately 5000-fold in mitochondria (21,22), concentrations on the inner mitochondrial membrane are likely to exceed 1 uM. Furthermore, prevention of MPT will help to sustain mitochondrial ATP production and improve islet viability. Other apoptosis inhibitors, such as caspase inhibitors or non–mitochondrial-targeted ROS scavengers, may reduce apoptosis but are unlikely to improve ATP production. Therefore, a major advantage of SS-31 is its targeted delivery to mitochondria and its ability to protect mitochondrial function.

Mouse islet cell transplantation studies generally use 500 to 600 islets that are isolated from 10- to 12-wk-old mice for prompt restoration of normoglycemia within 1 to 5 d after transplantation (44). Given that the successful reversal of hyperglycemia after syngeneic marginal islet cell transplants depends on a number of factors such as the presence of endotoxin in the islet preparation, degree of hyperglycemia, and even the strain of recipient mice (39,45,46), our islet graft recipients were paired for age, islet isolation reagents, and strain to control for variables that may affect transplant outcome. In our study, transplantation of 200 islets, isolated from >24-wk-old donor mice that were treated with SS-31, promptly restored normoglycemia (by day 1 after transplantation) in 50% of recipients. The observed survival benefit persisted despite discontinuation of SS-31 treatment on day 10 after transplantation. The ability to reverse hyperglycemia in this marginal mass transplant model suggests that the protection of islet mitochondrial potential and inhibition of apoptosis may translate in to improved posttransplantation function.

To date, the use of antioxidant treatment strategies to enhance islet transplant outcome has yielded limited benefits. These results may reflect the narrow therapeutic ranges of traditional antioxidants, the difficulty to achieve concentration levels that are high enough to reduce intracellular ROS, and their inability to target mitochondrial ROS generation.

In this regard, intraductal administration of glutamine has been shown to augment the number of human islets that are isolated and improve posttransplantation islet cell function in a marginal mass model (20). Glutamine’s beneficial effect has been attributed to its ability to increase the intracellular glutathione pool and thereby reduce cytosolic ROS. Whether intraductal delivery of SS-31 optimizes human islet isolation and improves posttransplantation function needs investigation.

In our investigation, the in vivo administration of SS-31 did not result in any observable increase in morbidity or mortality. Our finding of lack of toxicity extends earlier studies that demonstrated SS-31 and analogues to be nontoxic to heart or neuronal cells at dosages that ranged from 1 nM to 1 mM (21,22) and the data from Petri et al. (47) that in vivo administration to mice of dosages up to 50 mg/kg per d results in no obvious toxicity. Despite the lack of observable in vivo toxicity, additional studies are needed to determine whether posttransplantation islet function can be improved without systemic therapy and by in vivo treatment alone of islets with SS-31.

Conclusion

Our study demonstrates that the novel, antioxidant peptide SS-31 preserves mitochondrial membrane potential of isolated islets, inhibits apoptosis, optimizes islet yield, and improves islet graft function in recipients with diabetes. Mitochondria-targeted antioxidants may represent a new class of agents for optimizing islet isolation and transplantation.

Acknowledgments

This work was supported in part by an award from the Juvenile Diabetes Research Foundation (4-2001-924) and by awards from the National Institutes of Health: R21 DK60186 to M.S., PO1-DA08924 to H.H.S., and R21-NS-48295 to H.H.S.

We thank Dr. Peter Schiller (Clinical Research Institute of Montreal, Montreal, Quebec, Canada) for providing us with SS-31. [1H]SS-31 was synthesized by Dr. Peter Schiller and Dr. Geza Toth (Isotope Laboratory at the Institute of Biochemistry in the Biologic Research Centre, Szeged, Hungary). Dr. Thomas is a recipient of the Harold Amos Medical Faculty Development award from the Robert Wood Johnson Foundation.

Disclosures

Patent applications have been filed by Cornell Research Foundation (CRF) for the technology (SS peptides) described in this article. H.H.S. is the inventor. CRF, on behalf of Cornell University, has licensed the technology for further research and development to a commercial enterprise in which CRF and H.H.S. have financial interests.

References

6. Emamuallee JA, Shapiro AM: Interventional strategies to...


