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Coupling endoplasmic reticulum stress to cell death program in isolated human pancreatic islets: effects of gene transfer of Bcl-2

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Abstract A variety of toxic insults can result in endoplasmic reticulum (ER)-stress that ultimately leads to apoptosis. β-cells have a highly developed ER due to a great commitment to insulin production. The present study was carried out to determine the role of ER-stress in isolated human pancreatic islet apoptosis, and the potential protective effects of Bcl-2. Isolated human islets were infected with an adenoviral vector encoding Bcl-2 and then exposed to brefeldin-A, tunicamycin, A23187 and pro-inflammatory cytokines. Activation of caspase-12 was analyzed by means of Western blots. Apoptosis was evaluated using a commercial quantitative assay. ER-stress-inducers promoted caspase-12 activation and apoptosis, effect reversed by overexpression of Bcl-2. Co-localization of caspase-12 and Bcl-2 in the microsomal islet fractions were demonstrated by means of Western blots. We can conclude that the current studies highlight the importance of Bcl-2 as an anti-apoptotic protein, and shed new light on the mechanisms underlying its cytoprotective effects on pancreatic islets.

Keywords Islet transplantation · Endoplasmic reticulum · Bcl-2 · Apoptosis

Introduction

Recent dramatic progress in clinical outcome of pancreatic islet transplantation has been achieved using the "Edmonton Protocol" [1]. However, while performance of repeated islet infusions has fostered a major advance in insulin-free survival, the paucity of human islet supply relative to the surplus of diabetic patients demands that improved methods for achieving and maintaining functional islet mass are needed. To define strategies to preserve functional islet mass, it is essential to identify the mechanisms of ß-cell destruction and protection. Isolated human pancreatic islets are susceptible to myriad insults that occur during donor brain-death, organ procurement and preservation, isolation and transplantation [2, 3]. Although multiple mechanisms are involved in the reduction of functional islet mass, the common pathophysiological process is the trigger of the apoptotic machinery of β-cells. Thus, therapeutic targeting of the apoptotic pathways will be instrumental in facilitating islet transplantation as a cure for type I diabetes mellitus.

Proteases, particularly the family of cysteine proteases, have been proven to play critical roles in cellular execution of apoptosis. Most of them are located in the cytosol as zymogens, where they are activated by apoptotic stimuli-mediated signaling cascades. Caspase-8 mediates apoptotic signals from death receptors, and caspase-9 plays a key role in mitochondrial-mediated apoptosis. Downstream caspases, and executor caspases (such as caspases-3, 6 and 7), are activated next and are responsible for dismantling cellular proteins [4]. Recent investigations indicate that a variety of toxic insults, including $Ca2+$ ionophores, glucose deprivation,

chemical toxicants, nitric oxide (NO), and oxidative stress, can all cause endoplasmic reticulum (ER) stress and ultimately lead to apoptosis [5]. During activation of the ER-stress apoptotic pathway, caspase-12 is released into the cytoplasm to set in motion the cytosolic component of the apoptotic cascade, which involves, in part, activation of caspase-3 and caspase-7 [6].

A profound increase in proinflammatory cytokine release occurs after intraportal islet transplantation; this effect cannot be prevented using conventional immunosuppressive drugs. Proinflammatory cytokines increase the production of reactive oxygen species and NO, which lead to islet apoptosis and dysfunction [7]. Apoptosis is controlled through the expression of specific genes. Among these, the Bcl-2 gene family is one of the most important [8]. We previously demonstrated that overexpression of Bcl-2 in pancreatic islets prevents loss of functional islet mass after transplantation [9, 10]. Although Bcl-2 has a direct action on the mitochondrial membrane, it also resides and functions on the ER [11]. Based on increasing evidence for a role of the ER in apoptosis regulation, and the fact that ß-cells have a highly developed ER due to a heavy engagement in insulin production [12], this study was performed to investigate the role of the ER in islet apoptosis and the potential regulatory effect of Bcl-2 in isolated human islets.

Materials and methods

Pancreatic islet isolation

Pancreata were recovered from brain-dead cadaveric donors with informed consent. Islets were isolated by means of a semi-automatic method and purified using the Cobe 2991 as described [1]. The number of islets within each size class was converted to the standard number of islets of 150 µm diameter equal in volume to the sample. Purity was assessed by comparing the relative quantity of DTZ-stained endocrine tissue with unstained exocrine tissue. Islet viability was assessed in single islet cells following islet incubation at 37 °C for 17 min with 0.25% EDTA-trypsin (Sigma, St. Louis, Mo.) followed by syringe injection through progressively narrower gauge needles sized from 16–22 as described [13]. Then, islets cells were exposed to acridine orange (100 µg/ml) and ethidium bromide (100 µg/ml) and subjected to 2-color fluorescence microscopy. Viable cells were identified with green fluorescence, non-viable cells with red fluorescence. Approximately $2–5\times10^4$ cells were counted per islet isolation. Only islet preparations with >90% viability and >40% purity were used. The experiments were performed on islet preparations derived from a single donor in triplicate and repeated on at least 4 occasions on PI from subsequent donors.

Gene transfer

After isolation, groups of hand-picked islets were infected in 24-well tissue plates, in a minimum volume of 0.5 ml of medium [CMRL 1066 containing <2% fetal calf serum, 25 mM of HEPES and 100 U/ml of penicillin and 100 µg/ml of streptomycin (Mediatech, Herndon, VA)], at 37 °C, with an adenoviral vector encoding wild type human Bcl-2 (AdBcl-2) or an irrelevant gene (AdLacZ) at 500 pfu/cell as described [8, 14]. After 2 h, CMRL 1066 supplemented with 10% fetal calf serum was added and cultured overnight at 26 °C and 5% CO_2. After infection, islets were washed (three times) with CMRL 1066 medium and cultured for an additional 12 h before being tested for further experiments. Islet viability was assessed after the infection. Control islets were exposed to similar conditions without the infection. In an initial series of experiments, an adenovirus vector encoding green fluorescent protein (AdGFP) and β-galactosidase (LacZ) was used to determine the number of infected human islet cells. Following the infection, islets were dissociated into single cells and analyzed for GFP expression or LacZ by histochemical staining and microscopic quantitation. As previously reported in non-human primate islets [14], at MOI of 500, >95% of the human islet cells expressed the reported gene. These results were reproduced in islets from four different preparations. No significant differences in transfection efficiency were observed after islet infection with AdGFP, AdLacZ or Ad5Bcl-2. Similar results were observed in intact islets following immunohistological analysis of Bcl-2 expression in insulin producing cells [10]. We used AdLacZ as a vector control for the experiments because we observed a decrease in islet viability following infection with AdGFP.

Induction of ER-stress

Groups of 200–300 infected islets were placed in tissue culture plates containing CMRL 1066 containing 10% fetal calf serum, 25 mM of HEPES with 100 U/ml of penicillin and 100 µg/ml of streptomycin (Mediatech, Herndon, VA) at 37 °C, 5% CO_2. The effect of PIC was examined after culture for 2 days in human IFN-γ (1000 U/ml, R&D Systems, Minneapolis, Min.), IL-1β (50 U/ml, R&D Systems), and TNF-α (1000 U/ml, R&D Systems). IPHI were also exposed as indicated to Brefeldin-A (10 µg/ml), Tunicamycin (1 µg/ml), and A23187 (2 µM, Sigma), anti-Fas (J02, 4 µg/mL), and UV-radiation (2 mJ/cm^2 for 1 min). The Caspase-3 inhibitor Z-DEVD-FMK (75 µM, R&D Systems) was used as indicated 1 h before induction of apoptosis. Every day, fresh cytokines and drugs were added in concert with a medium change.

Fractionation experiments

In order to determine the site of action of caspase-12 and Bcl-2, cell fractions were prepared as described [6]. Briefly, islet samples were lyzed in buffer A (50 mM Tris-HCL pH 8.0, 1 mM β-mercaptoethanol, 1 mM EDTA, 0.32 M sucrose and 0.1 mM PMSF). Nuclear fraction was the first, 900 g, 10-min pellet, and mitochondrial fraction was the second, 5000 g, 10-min pellet, lysed by buffer A with an equal volume of 2×SDS buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 1.44 β-mercaptoethanol, 14% glycerol and 0.2% bromophenol blue). Microsomal and soluble fractions were extracted from the 105,000 g, 60-min pellet, and supernatant, respectively. Protein concentrations were determined using a Bio-Rad Protein assay kit I (Bio-Rad, Hercules, Calif.). Western blotting was carried out using polyclonal anti-caspase 12 antibody (Biovision Research Products, Mountain View, CA) at 1:500 dilutions, anti-caspase-3 (1:1000), Anti-PARP (1:1000, Cell Signaling Technology), anti-SRBP-1 (1:1000), anti-human Bcl-2 (1:500, Santa Cruz Biotechnology, Santa Cruz, Calif.), anti-cytochrome c (4CYTC-21, Trevigen, Gaithersburg, Md., at 1:1000), as described [6].

Apoptosis

The percentage of apoptotic islet cells was assessed using a quantitative assay (TiterTACS, Trevigen) following the manufacturer's

instructions. This assay allows the detection in situ of DNA fragmentation using colorimetric detection. The results are expressed as the mean percentage ± SEM of apoptotic cells.

Statistics

Treatment groups were compared by means of Student *t* test; significance was established at $P < 0.05$.

Results

Caspase-12 cleavage was examined after induction of apoptosis with different agents (Fig. 1A). Some cleavage of caspase-12 was demonstrated in untreated islets, probably reflecting ER-stress during isolation and culture. Apoptosis induced by anti-Fas antibody and UV-radiation did not increase caspase-12 cleavage. In contrast, agents known to induce ER-stress such as tunicamycin (which inhibits N-glycosylation in the ER) or the ionophore A23187 (disruption of the intracellular Ca2+ homeostasis) increased caspase-12 cleavage. Exposure of isolated human islets to proinflammatory cytokines also induced caspase-12 cleavage, an effect reproducible with the nitric oxide donor SNAP (data not shown) [15]. Since cleavage of caspases is a common indicator of activation, we conclude that caspase-12 can be specifically activated by apoptotic signals with an ER-stress component but not by apoptotic signals that do not induce ER-stress. To address the potential role of Bcl-2 on caspase-12 activation, islets were infected with an AdBcl-2 or AdLacZ and then exposed to proinflammatory cytokines. Islets that overexpressed Bcl-2 presented lower caspase-12 activation, compared with islets transfected with an irrelevant gene or uninfected

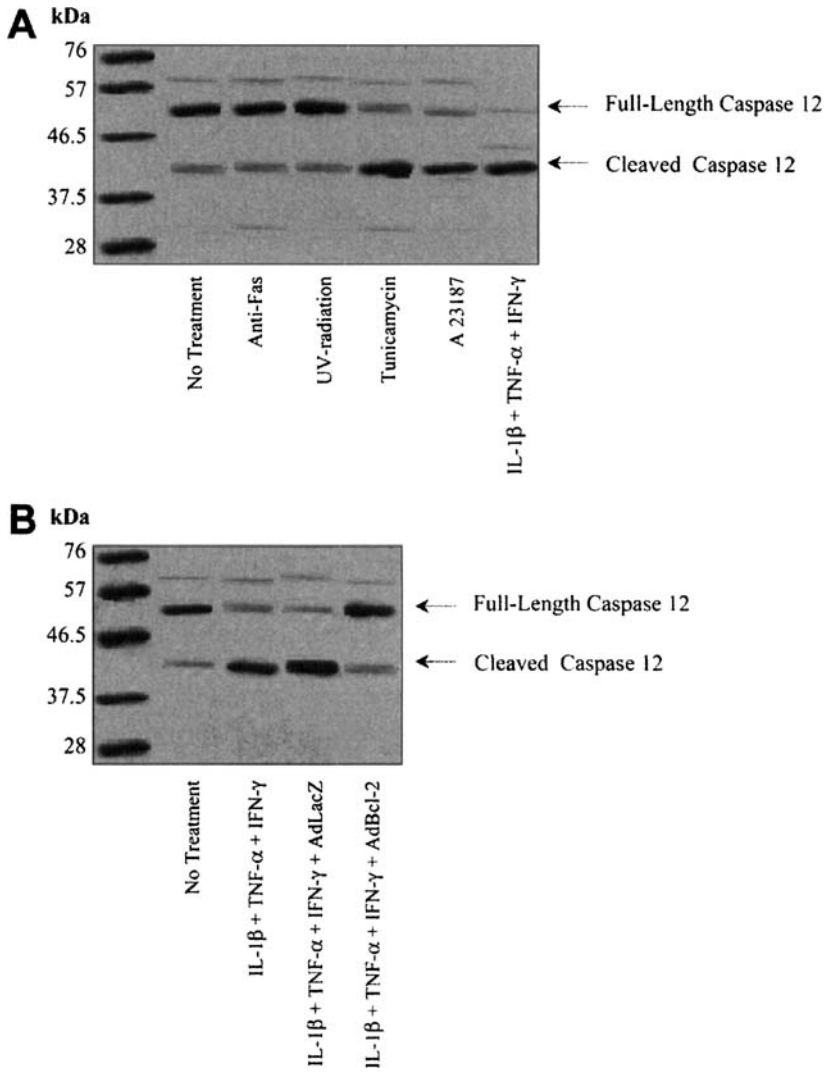

Fig. 1 **A** Specific caspase-12 activation by agents that induce ER-stress. Freshly isolated human islets were cultured and exposed to Anti-Fas antibody (4 μg/ml), UV-radiation, tunicamycin (1 μg/ml), A23187 (2 μM) and proinflammatory cytokines (IFN-γ, 1000 U/ml; IL-1β, 50 U/ml; TNF-α, 1000 U/ml) then analyzed by Western blot for Caspase-12 activation as described in Methods. **B** Bcl-2 prevents activation of caspase-12 after proinflammatory cytokines exposure. Isolated human islets were transfected with an adenovirus vector encoding Bcl-2 (AdBcl-2) or an irrelevant gene (AdLacZ) as described in Methods and exposed to proinflammatory cytokines. Caspase-12 activation was determined by Western blots. Figures are representative of three independent experiments

controls (Fig. 1B). Similar results were obtained after treatment with tunicamycin and A23187 (data not shown). These results demonstrate that gene transfer of Bcl-2 decreases ER-stress mediated apoptosis induced by agents that induce RE-stress, including proinflammatory cytokines.

To determine the site of action of caspase-12 and Bcl-2, lysates obtained from human islets were fractionated into nuclear, mitochondrial, microsomal and soluble fractions and analyzed by Western blots. As previously reported in other cell types [16], full-length caspase-12 was detected predominantly in the microsomal fraction (Fig. 2A), suggesting an association with the ER. As demonstrated in Fig. 1A, activation of caspase-12 occurs after islet isolation; cleaved caspase-12 was predominantly present in the soluble fraction. Caspase-3 was mainly detected in the mitochondrial and soluble fractions, but not in the microsomal fraction. Bcl-2 protein expression from AdBcl-2 infected islets was associated to the nuclear, mitochondrial, and microsomal fractions (Fig. 2B). In summary, these results demonstrate that caspase-12 is predominantly associated with ER where Bcl-2 also resides and functions.

To address the role of caspase-12 activation and Bcl-2 overexpression, we determined in vitro the percentage of apoptotic cells in islets infected with AdLacZ or AdBcl-2 and exposed to agents that induce ER-stress (Fig. 3). Brefeldin-A (an inhibitor of ER-Golgi transport), tunicamycin, A23187, and PIC induced significant activation of apoptosis, an effect partially reverted by the addition of Z-DEVD-FMK, an irreversible inhibitor of caspase-3. Further inhibition of apoptosis induced by these agents was demonstrated in islets infected with Ad-Bcl-2. The cytoprotective effect of Bcl-2 was dose-dependent. At MOI 500 (97.45 ± 3.78% of the islet cells transfected), AdBcl-2 reduced the apoptosis rate following brefeldin-A treatment to 10.02 ± 4.99%, Tunicamycin = 11.85 ± 6.28%, A23187 = 18.33 ± 6.11%, and proinflammatory cytokines = 10.44 ± 4.21% (Fig. 3), compared with AdLacZ infected controls (brefeldin-A = 47.98 ± 3.9%, Tunicamycin = 59.67 ± 3.1%, A23187 = 65.02 ± 5.13%, and proinflammatory cytokines = 54.29 ± 4.99%). Reducing the viral dose to MOI 100 (52.45 ± 3.90% of the cells were infected), AdBcl-2 infection reduced the apoptosis rate following brefeldin-A exposure to 29.3 ± 4.56%, Tunicamycin = 41.85 ± 5.78%, A23187 = 39.34 ± 5.98, and proinflammatory cytokines = 33.2 ± 4.77 (data not shown). Together, these results demonstrate that Bcl-2 prevents caspase-12 activation and therefore DNA fragmentation after islet exposure to agents that induce ER-stress, including proinflammatory cytokines. The fact that Z-DEVD-FMK partially reverted the apoptotic process induced by these agents indicates that activation of caspase-3 occurs after caspase-12 activation and is not the only pathway involved in ER-apoptosis [17].

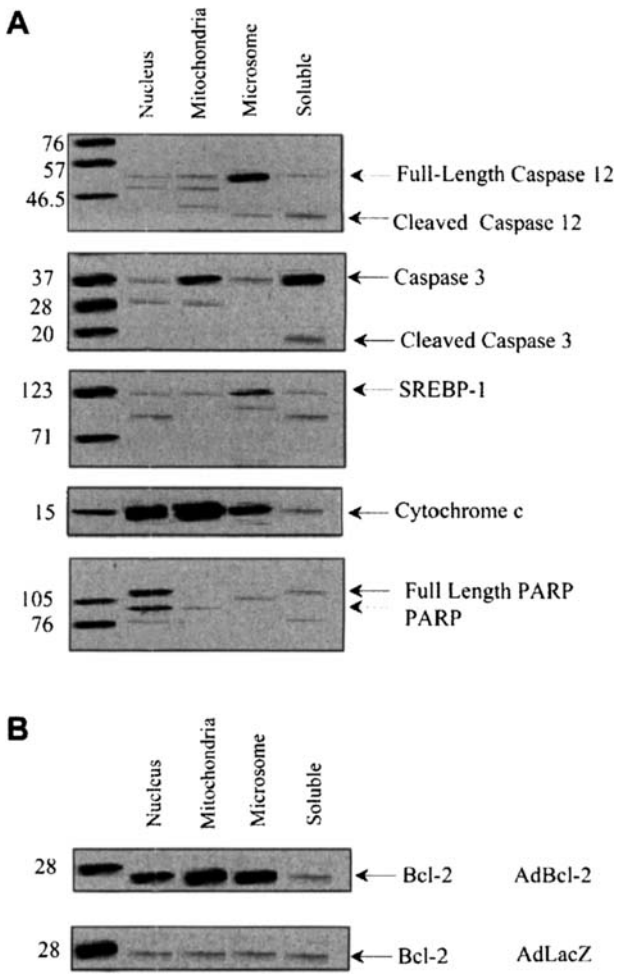

Fig. 2A, B Caspase-12 and Bcl-2 are co-localized in the ER. **A** Western blots of fractionated isolated islets were immunostained for caspase-12, caspase-3, SREBP-1, cytochrome c, and PARP as described in Methods. The quality of fractionations was controlled by the presence of known compartment-specific proteins analyzed by Western blots: SREBP-1 for the ER, cytochrome c for the mitochondria, and PARP for the nucleus. **B** Western blots of nuclear, mitochondrial, microsome and soluble fractions of IHPI infected with AdBcl-2 or AdLacZ. Figures are representative of three independent experiments

Discussion

The amount of transplanted islet mass is essential in the outcome of islet transplantation [1, 2, 3]. Thus, preventing loss of functional islet mass is crucial to facilitate islet transplantation as a cure for diabetes. Most secreted and integral membrane proteins are translocated cotranslationally into the lumen of the ER where post-translational modification, folding, and oligomerization occurs [5]. In addition, ER is the major signal-transducing

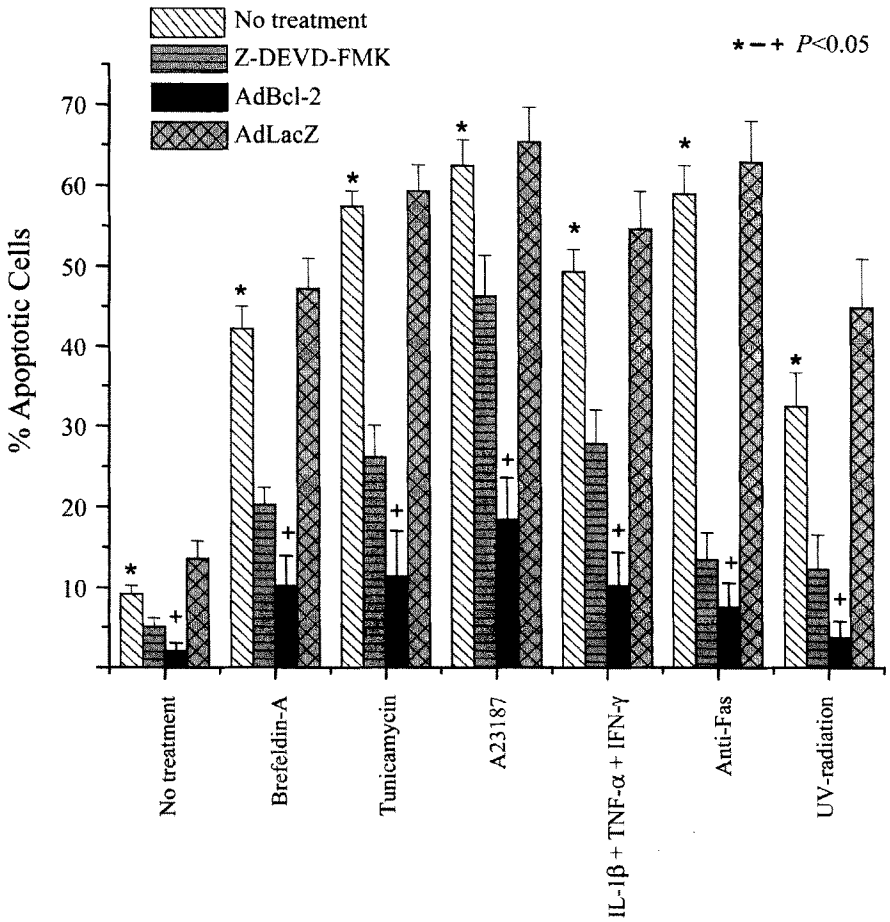

Fig. 3 Effect of Bcl-2 overexpression on isolated human islets exposed to ER-stress. Freshly isolated islets were infected with AdBcl-2 or AdLacZ and exposed to different agents that induce ER-stress as described in Methods. The caspase-3 inhibitor Z-DEVD-FMK was added as indicated 1-h prior to the induction of apoptosis. The percentage of apoptotic islet cells was assessed using a quantitative assay (TiterTACS, Trevigen) following the manufacturer's instructions. The results are expressed as the mean percentage ± SEM of apoptotic cells

organelle within the cell that continuously responds to environmental cues to release calcium. The ER is exquisitely sensitive to alterations in homeostasis, where, signals are transduced to the cytoplasm and the nucleus to eventually result in adaptation for survival or induction of apoptosis [18]. The early steps of insulin biosynthesis occur in the ER, and the β-cell has a highly developed and active ER. Recently, some forms of clinical and experimental diabetes have been linked to ER-stress [12].

Bcl-2 overexpression has been shown to prevent loss of functional islet mass [9, 10] and islet cell death after proinflammatory cytokine exposure [19, 20]. We have demonstrated in the present study a co-localization of Bcl-2 with Caspase-12 in the ER. For the first time, we report here that overexpression of Bcl-2 decreased the activation of caspase-12 and protected isolated human islets against ER-stress mediated apoptosis. The mechanisms of Bcl-2 prevention of caspase-12 activation are currently under study in our laboratory. Several potential mechanisms may be involved: Bcl-2 has been shown to regulate intracellular Ca^{2+} concentrations [21] and decrease the activation of caspases [8, 11]. Another potential mechanism includes the inhibition of cytochrome c release from the mitochondria after brefeldin-A and tunicamycin treatment, as previously reported [22]. Thus, apoptotic agents perturbing ER functions may induce a crosstalk between the ER and the mitochondria that can be interrupted by ER-resident Bcl-2. It is clear that further study is needed in order to elucidate these signaling mechanisms.

The current studies highlight the importance of Bcl-2 as an anti-apoptotic protein and shed new light on the mechanisms underlying its cytoprotective effects on pancreatic islets. The results could have broad implications for our understanding of the mechanisms by which pancreatic islets couple ER-stress to the cell death program and by which functional islet mass is lost after islet isolation and transplantation.

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