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Short-Term Culture With the Caspase Inhibitor z-VAD.fmk Reduces Beta Cell Apoptosis in Transplanted Islets and Improves the Metabolic Outcome of the Graft

Marta Montolio, Noèlia Téllez, Montserrat Biarnés, Joan Soler, and Eduard Montanya

Laboratory of Diabetes and Experimental Endocrinology, Endocrine Unit, IDIBELL-Hospital Universitari de Bellvitge, University of Barcelona, Barcelona, Spain

In the initial days after transplantation islets are particularly vulnerable and show increased apoptosis and necrosis. We have studied the effects of caspase inhibition on this early beta cell death in syngeneically transplanted islets. Streptozotocin-diabetic C57BL/6 mice were transplanted with 150 syngeneic islets, an insufficient mass to restore normoglycemia, preincubated with or without the pan-caspase inhibitor z-VAD.fmk 2 h before transplantation. Beta cell apoptosis was increased in control islets on day 3 after transplantation ($0.28 \pm 0.02\%$) compared with freshly isolated islets ($0.08 \pm 0.02\%$, $p < 0.001$), and was partially reduced in transplanted islets preincubated with z-VAD.fmk 200 μM ($0.14 \pm 0.02\%$, $p = 0.003$) or with z-VAD.fmk 500 μM ($0.17 \pm 0.01\%$, $p = 0.012$), but not with a lower z-VAD.fmk (100 μM) concentration. Diabetic mice transplanted with islets preincubated with z-VAD.fmk 500 μM showed an improved metabolic evolution compared with control and z-VAD.fmk 200 μM groups. The z-VAD.fmk 500 μM group showed an overall lower blood glucose after transplantation ($p = 0.02$), and at the end of the study blood glucose values were reduced compared with transplantation day (15.7 ± 3.6 vs. 32.5 ± 0.5 mmol/L, $p = 0.001$). In contrast, blood glucose was not significantly changed in control and z-VAD.fmk 200 μM groups. Four weeks after transplantation beta cell mass was higher in z-VAD.fmk 500 μM group (0.15 ± 0.02 mg) than in the control group (0.10 ± 0.02 mg) ($p = 0.043$). In summary, the treatment of freshly isolated islets with the caspase inhibitor z-VAD.fmk reduced the subsequent apoptosis of the islets once they were transplanted and improved the outcome of the graft.

Key words: Islet transplantation; Apoptosis; Caspase; z-VAD.fmk; Beta cell mass

INTRODUCTION

Transplanted islets are particularly vulnerable in the initial days after transplantation (5), and we recently reported that, even in syngeneic grafts, more than 60% of transplanted islet mass was lost due to increased apoptosis and necrosis (2). Several factors may play a role in the initial death of islets, including damage during isolation (23,29), technical problems in the transplantation procedure (12), islet hypoxia (6), absence of survival factors present in the nonendocrine pancreas (10), disruption of islets cellular connections to extracellular matrix (27), or nonspecific inflammation at the grafted site (21). This early islet death can contribute to increase the beta cell mass required to achieve normoglycemia in diabetic recipients and can have a negative effect on the long-term evolution of the graft after transplantation. Thus, it may be expected that a reduction in the initial beta cell death would improve the outcome of the graft. However, this has not been directly determined. Moreover, because initial apoptosis after transplantation is low in absolute terms it is not known whether a reduced apoptosis would modify the evolution of the graft.

Caspases are the central executioners of the apoptotic process (8) and accordingly caspase activation has been found in beta cells undergoing apoptosis (33). These enzymes participate in a cascade triggered in response to apoptotic signals that end in cleavage of proteins, leading to disassembly of the cell (28). Caspase inhibitors (19,30) have been shown to reduce apoptotic cell death both in vitro (11,25) and in vivo (3,4,9). Notably, caspase inhibition reduced apoptosis in nigral transplants and increased the number of surviving dopaminergic cells (24). In human islets the pan-caspase inhibitor, z-

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Address correspondence to Eduard Montanya, Endocrine Unit (13-2), IDIBELL-Hospital Universitari Bellvitge, Feixa Llarga, s/n, 08907 L'Hospitalet de Llobregat, Barcelona. Spain. E-mail: montanya@ub.edu

Val-Ala-DL-Asp-fluoromethylketone (z-VAD.fmk), reduced beta cell death in human beta cells in vitro (1,22). However, the in vivo effect of caspase inhibition on islet cell death has not been determined. The aim of this study was to determine whether exposure of isolated islets to the pan-caspase inhibitor z-VAD.fmk before transplantation could reduce or prevent the initial apoptotic beta cell death after transplantation and whether this reduction could improve the outcome of the graft.

MATERIALS AND METHODS

Animals

Male inbred C57Bl/6 mice (Harlan France SARL, Gannat, France) aged 8–12 weeks, were used as donors and recipients of transplantation. The recipients were made diabetic by a single IP injection of streptozotocin (STZ) (Sigma, St. Louis, MO) 180 mg/kg body weight, freshly dissolved in citrate buffer (pH 4.5). Transplantation was performed 7–14 days after STZ injection. Before transplantation, diabetes was confirmed by the presence of hyperglycemia, weight loss, and polyuria. Only those mice with a blood glucose higher that 20 mmol/L on a minimum of two consecutive measurements were transplanted. Blood glucose was determined between 0900 and 1100 h in nonfasting conditions. Blood was obtained from the snipped tail and glucose was measured with a portable glucose meter (Glucocard Memory, A. Menarini Diagnostics, Barcelona, Spain). Animals were kept under conventional conditions in climatized rooms with free access to tap water and standard pelleted food.

Experimental Protocol

The ability of different concentrations of the nonselective inhibitor of caspase activation, z-VAD.fmk (Bachem, Bubendorf, Switzerland), to reduce apoptosis in transplanted beta cells was initially tested on day 3 after transplantation. Islets were preincubated for 2 h with z-VAD.fmk 100 μM ($n = 3$), 200 μM ($n = 6$), 500 μM ($n = 6$), or with no z-VAD.fmk (control group, $n = 9$), and were transplanted under the kidney capsule of the recipient. z-VAD.fmk was prepared in dimethyl sulphoxide (DMSO, Sigma). In all experimental conditions, including the control group, the final concentration of DMSO in the culture media was kept at 0.05%. Three days after transplantation, the grafts were harvested and beta cell apoptosis was determined.

The z-VAD.fmk concentrations that were found to induce a reduction in transplanted beta cell apoptosis were then subsequently used to analyze the effects on metabolic evolution. One hundred and fifty freshly isolated islets were preincubated for 2 h before transplantation with z-VAD.fmk 200 μM ($n = 10$), z-VAD.fmk 500 μM ($n = 7$), or DMSO 0.05% (control group, $n = 15$) and were transplanted into STZ-diabetic recipients. One hundred and fifty islets is an insufficient beta cell mass to restore normoglycemia in this model and therefore animals were expected to remain hyperglycemic (16). Grafts were harvested 4 weeks after transplantation and beta cell mass was evaluated. Blood glucose and body weight were determined on days 3 and 7 after transplantation, and weekly thereafter. Animals were considered normoglycemic when blood glucose values after transplantation were <8.9 mmol/L (mean + 2 SD of all animals included in the study before streptozotocin injection).

Islet Isolation, Transplantation, and Graft Harvesting

Islets were isolated by collagenase (Collagenase P, Boehringer Mannheim Biochemicals, Mannheim, Germany) digestion of the pancreas as previously described (18). Isolated islets were hand-picked under stereomicroscope two or three times, until a population of pure islets was obtained. To homogenize the islet population among groups, and to reduce the effect of hypoxia on transplanted islets, only those islets >75 and <250 μm in diameter were used for transplantation.

After isolation, islets were counted into groups of 150 islets and cultured at 37°C and 5% CO_2 for 2 h in RPMI-1640 medium supplemented with 11.1 mM glucose and 10% fetal calf serum (FCS, Biological Industries, Beit Haemek, Israel) and with z-VAD.fmk 100, 200, or 500 μM or with DMSO 0.05% with no z-VAD.fmk (control group). Immediately after the 2-h culture, islets were transplanted under the left kidney capsule of mice recipients. After transplantation, the capsulotomy was cauterized with a disposable low-temperature cautery pen (AB Medica, S.A., Barcelona), and the lumbar incision was sutured (18).

Grafts were harvested under the microscope as previously described (2), and after the retrieval of the graft the kidney cortex was carefully examined to ensure that no islet tissue was left in place. The graft was immediately immersed in 4% paraformaldehyde fixative and processed for paraffin embedding.

Apoptosis Detection

Graft sections (2 μm) were double-stained by immunoperoxidase for apoptotic nuclei using the deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique (In Situ Cell Death Detection Kit, ApopTag®, Intergene, Oxford, UK), and by alkaline phosphatase for the endocrine non-beta cells of the islets, as described (2). We stained the endocrine non-beta cells instead of beta cells because the severe hyperglycemia that was expected is associated with beta cell degranulation, resulting in negative or weak staining. After immunoperoxidase staining, beta cells and apoptotic nu-

clei were 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. Beta cell apoptosis was expressed as percentage of TUNEL-positive beta cells. A minimum of 1000 cell nuclei were counted per graft; the sections were systematically sampled, all endocrine nuclei were counted, and a second section was included when needed.

Apoptosis in islets before transplantation was determined in seven groups of 150 islets isolated on different days. After isolation, the islets were washed in phosphate-buffered saline (PBS) and pelleted in 4% paraformaldehyde fixative. Sections of the islet pellet were double-stained for TUNEL and for endocrine non-beta cells, and beta cell apoptosis was expressed as percentage of TUNEL-positive beta cells. In addition, beta cell apoptosis in normal pancreas was determined in eight pancreas 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 beta cell apoptosis, sections of the pancreas were double-stained and counted as described for grafts and isolated islets.

Islet Cell Necrosis

The area of necrosis was measured by point counting morphometry (32) on the same graft sections used to quantify beta cell mass. The necrotic area was expressed as percentage of intercepts over necrotic tissue divided by intercepts over islet tissue (beta and non-beta cells) and necrotic area (2).

Beta Cell Mass

Methods used for measurement of beta cell mass have been described in detail (16,18). Beta cell mass of islet grafts was measured by point counting morphometry on 3-μm sections using a 48-point grid to obtain the number of intercepts over beta cells, over endocrine non-beta cells, and over other tissue. The beta cell relative volume was calculated by dividing the intercepts over beta cells by intercepts over total tissue; then beta cell mass was estimated by multiplying beta cell relative volume by graft weight.

The beta cell mass of islets at the time of transplantation was determined in 10 groups of 150 isolated islets, and the beta cell mass after 3-day culture in RPMI-1640 medium supplemented with 11.1 mM glucose and 10% FCS was determined in nine groups of 150 islets. 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. Beta cell mass was obtained by multiplying the weight of the islets by the percentage of beta cell volume, determined with image-analytical software (AnalySIS 3.0; Soft Imaging System, Münster, Germany) on sections of the islet pellets.

Statistical Analysis

Results were expressed as mean ± SEM. Differences between means were evaluated using the one-way analysis of variance (ANOVA) or the Kruskal-Wallis test as appropriate. The Fisher's PLSD (protected least significant difference) method or the Mann-Whitney test were used to determine specific differences between means when determined as significant by ANOVA main effects analysis or by Kruskal-Wallis test, respectively. The statistical software SPSS 10.0 was used. A value of $p < 0.05$ was considered significant.

RESULTS

Dose of z-VAD.fmk That Effectively Reduces Apoptosis in Transplanted Islets

To identify which dose of the caspase inhibitor was capable to reduce apoptosis in transplanted islets, we transplanted 150 syngeneic islets previously cultured with 100, 200, and 500 μM z-VAD.fmk. Grafts were harvested 3 days later and beta cell apoptosis was analyzed.

On day 3 after transplantation, beta cell apoptosis was significantly increased in the control group (0.28 ± 0.02%) compared with freshly isolated islets (0.08 ± 0.02%, $p < 0.001$) and with normal pancreas (0.05 ± 0.02%, $p < 0.001$) (Fig. 1). Beta cell apoptosis in grafts of islets preincubated with z-VAD.fmk 100 μM was not modified (0.34 ± 0.08%). However, preincubation with z-VAD.fmk 200 and 500 μM before transplantation reduced subsequent beta cell apoptosis in graft islets by 50% (0.14 ± 0.02%, $p = 0.003$) and 40% (0.17 ± 0.01%, $p = 0.012$), respectively. On the other hand, the large necrotic areas found on day 3 after transplantation were unaffected by caspase inhibition (control group: 23.6 ± 9.6%; z-VAD.fmk 200 μM group: 27.8 ± 10.7%; z-VAD.fmk 500 μM group: 17.5 ± 6.1%), suggesting that reduced apoptosis did not result in increased necrotic cell death. On day 30, no necrosis was found in any graft.

Effect of z-VAD.fmk on Islet Graft Evolution

Metabolic Evolution of Transplanted Animals. To determine whether reduction in beta cell apoptosis could improve graft survival we transplanted a marginal beta cell mass (150 islets) into diabetic syngeneic recipients and monitored the metabolic evolution for 4 weeks. Before transplantation islets were preincubated with z-VAD.fmk 200 or 500 μM, the concentrations that we had found to reduce beta cell apoptosis on day 3 after transplantation.

Blood glucose and body weight were similar in all groups when injected with STZ as well as when trans-

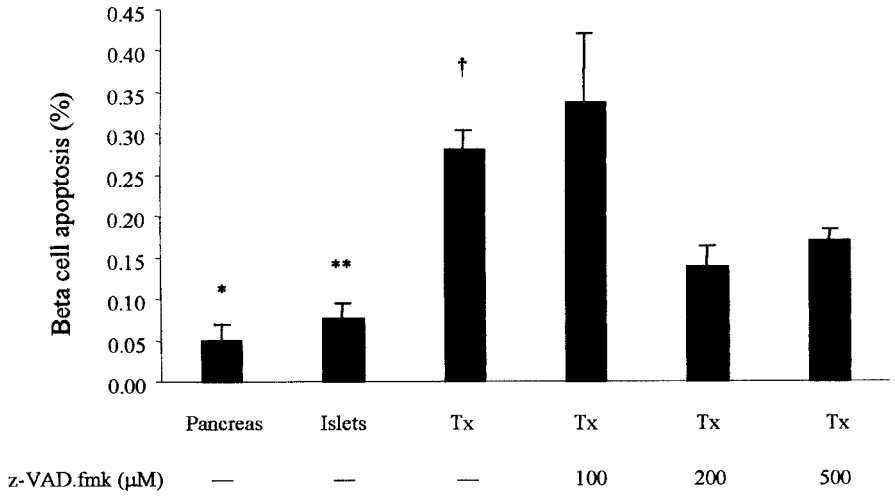

Figure 1. Beta cell apoptosis in pancreas ($n = 8$), freshly isolated islets ($n = 7$), and islet grafts on day 3 after transplantation. Islets in transplanted groups (Tx) were preincubated for 2 h in the absence of z-VAD.fmk ($n = 9$) or with z-VAD.fmk 100 μM ($n = 3$), 200 μM ($n = 6$), and 500 μM ($n = 6$). Apoptosis was expressed as percentage of apoptotic nuclei over beta cells. Values are means ± SEM. Kruskal-Wallis, $p < 0.001$; Mann-Whitney: $*p < 0.05$ pancreas versus all other groups but islets. $**p < 0.02$ islets versus all other groups but pancreas and Tx 200 μM z-VAD.fmk. $\dagger p < 0.02$ Tx versus Tx 200 and 500 μM z-VAD.fmk.

planted (Table 1). Because 150 islets is an insufficient mass to restore normoglycemia (16), most of the animals (73.4%) transplanted with control islets remained hyperglycemic 4 weeks after transplantation. In the two groups transplanted with islets preincubated with 200 or 500 μM z-VAD.fmk, 70% and 57% of the recipients remained hyperglycemic 4 weeks after transplantation, respectively. When blood glucose evolution after transplantation (from day 3 to day 28) was considered, blood glucose was different among the three groups (Kruskal-Wallis, $p = 0.02$), with lower values in the z-VAD.fmk 500 μM group (21.2 ± 1.5 mmol/L) than in the control (25.3 ± 1.1 mmol/L, $p = 0.008$) and z-VAD.fmk 200 μM (25.2 ± 1.2 mmol/L, $p = 0.032$) groups (Fig. 2A). Four weeks after transplantation, blood glucose values were reduced in the z-VAD.fmk 500 μM group (15.7 ± 3.6 mmol/L) compared with transplantation day (32.5 ± 0.5 mmol/L, $p = 0.001$), but were not significantly different in the control (22.8 ± 3.0 vs. 31.0 ± 0.9 mmol/L) and z-VAD.fmk 200 μM (22.6 ± 3.9 vs. 32.9 ± 0.2 mmol/L) groups. Body weight after transplantation showed a similar evolution as blood glucose, but differences did not reach statistical significance (Fig. 2B).

Beta Cell Mass in Transplanted Islets. Beta cell mass in transplanted islets preincubated with 200 or 500 μM z-VAD.fmk, or with DMSO 0.05% was determined on days 3 and 28 after transplantation. The initially transplanted beta cell mass, measured in 150 freshly isolated islets (0.29 ± 0.02 mg), was reduced in all three groups on day 3 after transplantation: control group (0.10 ± 0.02 mg), z-VAD.fmk 200 μM (0.07 ± 0.01 mg),

Table 1. Characteristics of Experimental Groups

		Streptozotozin Injection		Transplantation		Graft Removal (4 Weeks After Transplantation)	
Group	*n*	Blood Glucose (mmol/L)	Body Weight (g)	Blood Glucose (mmol/L)	Body Weight (g)	Blood Glucose (mmol/L)	Body Weight (g)
Control (DMSO 0.05%)	15	6.9 ± 0.2	25.0 ± 0.2	31.0 ± 0.9	23.3 ± 0.4	22.8 ± 3.0	24.5 ± 0.6
z-VAD.fmk 200 μM	10	6.8 ± 0.5	24.8 ± 0.3	33.0 ± 0.2	23.0 ± 0.6	22.6 ± 3.9	25.0 ± 0.5
z-VAD.fmk 500 μM	7	7.1 ± 0.4	24.7 ± 0.2	32.5 ± 0.5	23.4 ± 0.4	15.7 ± 3.6	25.7 ± 0.5

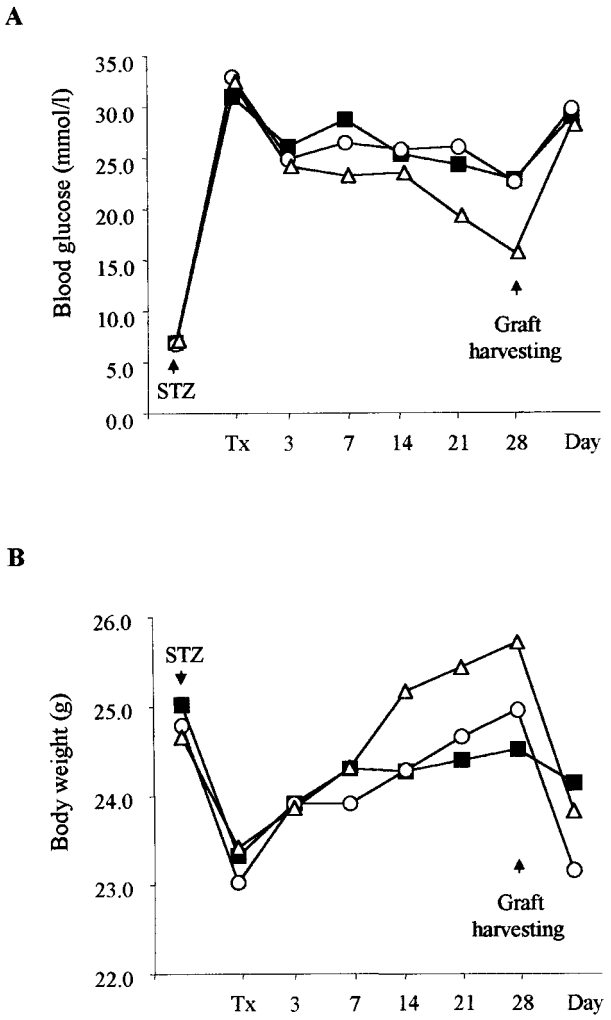

Figure 2. Evolution of blood glucose (A) and body weight (B) in control group (■), z-VAD.fmk 200 μM group (○), and z-VAD.fmk 500 μM group (△). STZ: day of streptozotocin injection. Tx: day of islet transplantation.

and z-VAD.fmk 500 μM (0.09 ± 0.01 mg) ($p < 0.001$) (Fig. 3). In contrast, 3 days of culture did not modify the beta cell mass of 150 islets (0.27 ± 0.04 mg), indicating that the beta cell mass reduction found in day 3 grafts was due to transplantation, not to the fact that islets were 3 days old. Although the grafted beta cell mass was still reduced in all three transplanted groups 4 weeks after transplantation, it had increased in animals transplanted with islets preincubated with 500 μM z-VAD.fmk, and was higher than in the control group (control: 0.10 ± 0.02 mg; z-VAD.fmk 500 μM: 0.15 ± 0.02 mg, $p = 0.043$).

DISCUSSION

The massive beta cell death that takes place in the initial days after transplantation plays a major role in early graft failure. This early beta cell loss increases the islet number required for successful islet transplantation, and it can also limit the long-term survival of the graft, which has been shown to be dependent on the initially transplanted beta cell mass (13,14,31). The understanding of the events that take place in the crucial initial days after transplantation is limited, but we have shown the contribution of both necrosis and apoptosis to early beta cell death in transplanted islets (2). On day 3 after transplantation, extensive areas of necrosis (about 30% of the transplanted islet mass) were identified in the graft, and beta cell apoptosis was 10-fold higher than in normal pancreatic beta cells. However, the number of apoptotic beta cells was still quite low, 0.3–0.4% of the whole beta cell population in the graft, and the significance of this increased beta cell apoptosis to the graft outcome could be questioned. We have hypothesized that because apoptosis is a very rapid process, the detection of this higher frequency of apoptotic beta cells reflected in fact an important increment in total beta cell apoptosis, sufficient to significantly reduce beta cell mass (2). The finding, in the current study, that even a partial reduction in early apoptosis was able to improve the outcome of the graft confirms the biological significance of the increased beta cell apoptosis in early islet transplantation.

The effects of z-VAD.fmk on beta cell apoptosis in transplanted islets were dose dependent. The 100 μM concentration, which was sufficient to reduce cytokine-induced beta cell apoptosis in mouse islets in vitro (17), did not reduce beta cell apoptosis when islets were subsequently transplanted. A higher concentration (200

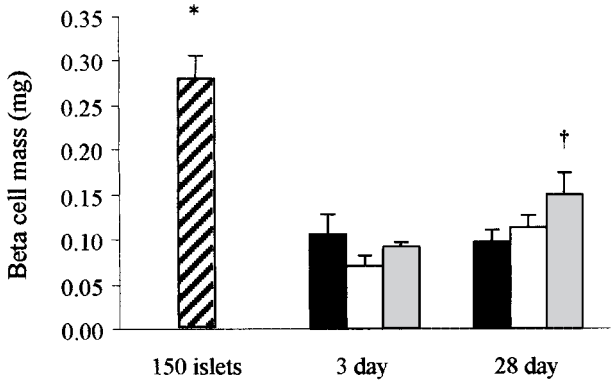

Figure 3. Beta cell mass in 150 isolated islets, and islet grafts on days 3 and 28 after transplantation Black bars: control islets; white bars: islets preincubated (2 h) with z-VAD.fmk 200 μM before transplantation; gray bars: islets preincubated (2 h) with z-VAD.fmk 500 μM before transplantation. Values are means ± SEM. ANOVA, $p < 0.001$; Fisher PLSD: $^*p < 0.001$ 150 freshly isolated islets versus all other groups. $^\dagger p < 0.05$ control versus z-VAD.fmk 500 μM on day 28.

μM) achieved a significant reduction in transplanted beta cell apoptosis but was, however, insufficient to modify the metabolic evolution of the recipient. Eventually, the incubation of islets with a 500 μM zVAD.fmk resulted in both the reduction in transplanted beta cell apoptosis and the improvement in the metabolic outcome after transplantation. A similar dose dependency in caspase inhibition of apoptosis has been shown in serum deprivation-induced neuronal death (24). Moreover, high concentrations of the caspase inhibitor Ac-YVAD.cmk (500 μM) were successfully used in nigral transplants (24). The requirement of high concentrations of caspase inhibitor in order to achieve an effective inhibition of islet apoptosis in transplanted islets, particularly compared with the lower concentrations that have been useful in vitro, may reflect the multiplicity and intensity of the stimulus contributing to beta cell death in the early days after transplantation.

Despite the significant reduction in beta cell apoptosis and the improved metabolic outcome, beta cell mass was still significantly reduced initially after transplantation, and 57% of the recipients transplanted with islets preincubated with z-VAD.fmk 500 μM remained hyperglycemic. Even though beta cell apoptosis was reduced by 50% in islets cultured with z-VAD.fmk before transplantation, apoptosis was still considerably higher than in normal pancreas, and sufficient to result in significant beta cell death. In addition, it has been shown that inhibition of caspase activation cannot rescue those cells that have been irreparably damaged and that may end up dying, even though more slowly and without the morphological and biochemical markers of apoptosis (7). Death from nonapoptotic programmed cell death has been described after the use of pharmacological modulators of apoptotic pathway in neurodegenerative processes (26). Finally, necrosis, an important contributor to transplanted beta cell death, was unaffected by caspase inhibition, suggesting that it was not secondary to the large amount of apoptosis in the graft. All these factors may have contributed to the persistence of a severe beta cell mass reduction in the initial days after transplantation in z-VAD.fmk-treated islets and to limit the beneficial effects of caspase inhibition. Nevertheless, it is noteworthy that, considering all this limitations, the reduction in beta cell apoptosis after this short-term incubation with z-VAD.fmk was able to increase the graft beta cell mass and to improve the long-term metabolic outcome.

Several strategies, both before and after transplantation, have been reported to improve the outcome of experimental islet transplantation. However, the results of the interventions have been usually determined based solely on changes in the metabolic condition of the recipient. This limited assessment may miss fundamental information about the effectiveness of the intervention that can be obtained if graft parameters such as beta cell mass, replication, or death are measured. For instance, we have shown that maintaining normoglycemia in the initial days after transplantation improves the outcome of the graft and reduces the beta cell mass required to achieve normoglycemia (15). However, the quantification of grafted beta cell mass unexpectedly disclosed that normoglycemia did not preserve the transplanted beta cell mass in the early days after transplantation, and that the beneficial effect was probably due to the preservation of beta cell function in transplanted islets maintained in normoglycemic conditions (15,20). In the current experiments, the measurement of beta cell apoptosis in islet grafts revealed the inhibitory effects of z-VAD.fmk 200 μM, a beneficial effect that would have been missed if only the metabolic evolution had been determined. This beneficial effect was then confirmed by the improved metabolic evolution found when z-VAD.fmk 500 μM was used, but also, and importantly, by the higher beta cell mass in the graft.

In summary, we have found that the incubation of freshly isolated islets with an appropriate dose of the caspase inhibitor z-VAD.fmk reduced beta cell apoptosis when islets were subsequently transplanted. This reduction improved the metabolic evolution of the graft and partially preserved the transplanted beta cell mass, indicating the biological significance of initial beta cell death, and in particular of beta cell apoptosis, on the long-term outcome of the graft. Our results confirm that there is a window for therapeutic intervention on isolated islets, before they are transplanted, that can be used to modify the characteristics of the islets and to improve the outcome of the graft.

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