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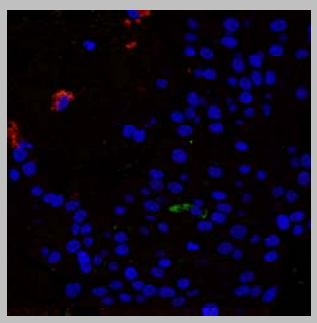

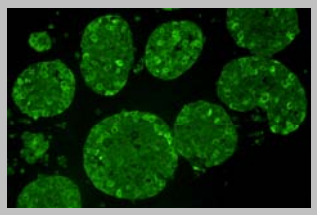

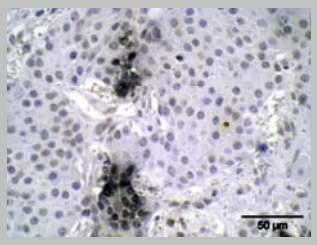

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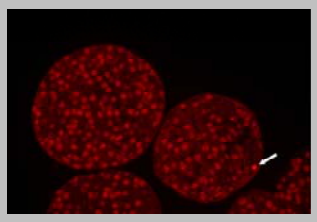

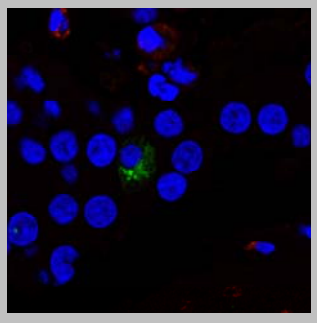

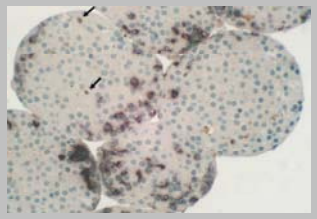

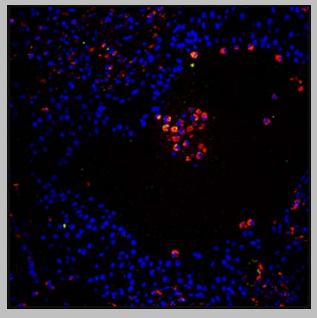

Marta Montolio Rusiñol

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PRODUCCIÓ CIENTÍFICA

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Montolio M, Téllez N, Biarnés M, Soler J, Montanya E. 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. Cell Transplantation 14:59–65, 2005.

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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

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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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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-

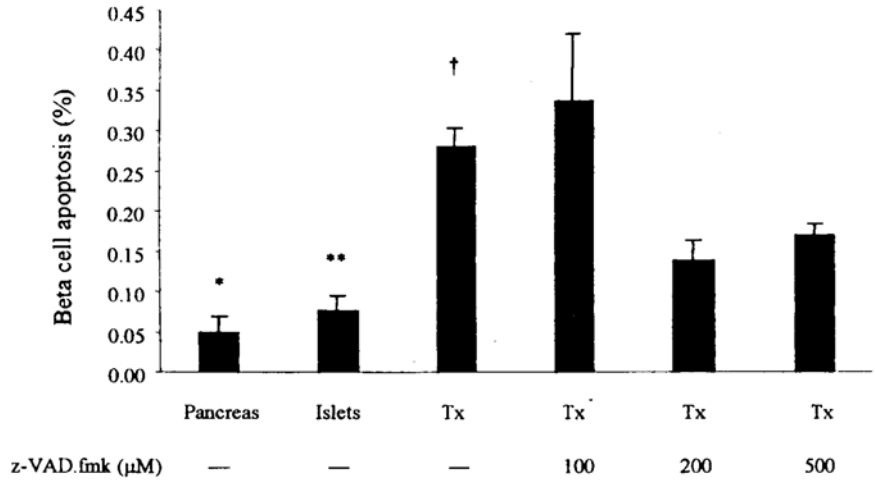

Figure 1. Beta cell apoptosis in pancreas ($n = 8$), freshly isolated islets ($n = 7$), and islet grafts on day 3 after transplantation. Islets in transplanted groups (Tx) were preincubated for 2 h in the absence of z-VAD.fmk ($n = 9$) or with z-VAD.fmk 100 μM ($n = 3$), 200 μM ($n = 6$), and 500 μM ($n = 6$). 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

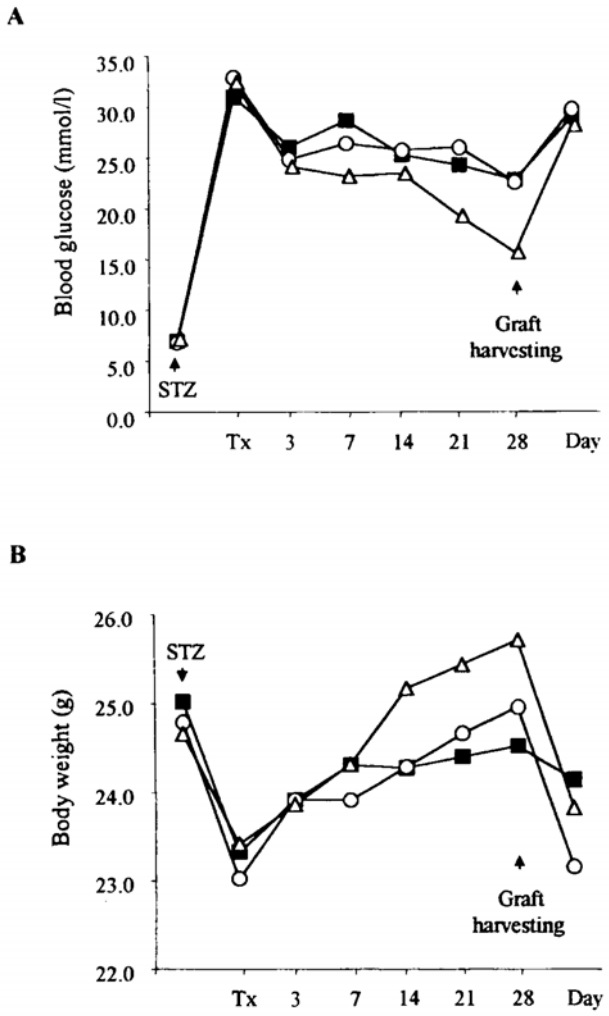

Figure 2. Evolution of blood glucose (A) and body weight (B) in control group (■), z-VAD.fmk 200 μM group (○), and z-VAD.fmk 500 μM group (△). STZ: day of streptozotocin injection. Tx: day of islet transplantation.

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

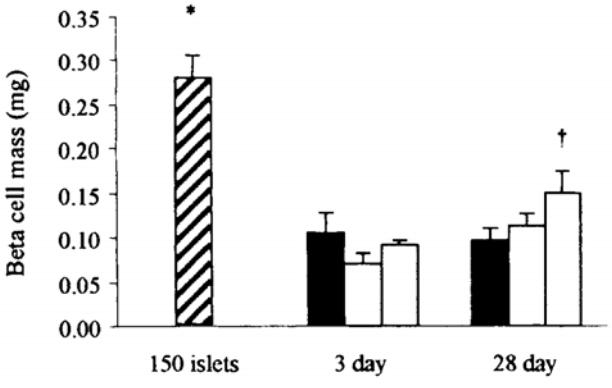

Figure 3. Beta cell mass in 150 isolated islets, and islet grafts on days 3 and 28 after transplantation Black bars: control islets; white bars: islets preincubated (2 h) with z-VAD.fmk 200 μM before transplantation; gray bars: islets preincubated (2 h) with z-VAD.fmk 500 μM before transplantation. Values are means ± SEM. ANOVA, $p < 0.001$; Fisher PLSD: *$p < 0.001$ 150 freshly isolated islets versus all other groups. †$p < 0.05$ 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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SUGGESTED RUNNING HEAD: IL-1β and iNOS in islet grafts

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ABSTRACT

Islets are particularly vulnerable in the initial days after transplantation when cell death results in the loss of more than half of the transplanted islet tissue. To determine whether a non-specific inflammation at the grafted site mediated by the local expression of inflammatory cytokines could play a role on the initial damage to transplanted islets we studied the expression of IL-1β and iNOS after syngeneic islet transplantation. Insulin-treated streptozotocin-diabetic Lewis rats were syngeneically transplanted with 500 islets. Grafts were harvested 1, 3 or 7 days after transplantation, and the expression of IL-1β and iNOS genes was determined by RT-PCR. IL-1β and iNOS mRNA was detected in islets immediately after isolation, and was up-regulated after transplantation. IL-1β mRNA was 9-fold increased on day 1, was still 7-fold increased on day 3 after transplantation and declined towards pre-transplantation levels on day 7. iNOS mRNA showed a similar pattern of expression than IL-1β: on days 1 and 3 after transplantation it was 14- and 4-fold higher respectively than in freshly isolated islets. In addition, IL-1β and iNOS were identified in islet grafts and found to be produced mainly by CD68-positive macrophages. A low number of IL-1β- and iNOS-positive but CD68-negative cells was also identified suggesting that other cell types, in addition to macrophages, were involved in the expression of IL-1β and NO production in islet grafts. The finding of an increased IL-1β and iNOS gene expression in the initial days after islet transplantation and the presence of IL-β and iNOS proteins in the graft, confirmed the presence of an early non-specific inflammatory response after islet transplantation. Overall, the data suggest that IL-1β plays a role in the extensive beta cell death found in the initial days after islet transplantation.

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Islet transplantation is limited by the insufficient supply of islet tissue, a problem that is further aggravated by the high number of islets required for successful transplantation (Ryan *et al.* 2005). Islets are particularly vulnerable in the initial days after transplantation (Davalli *et al.* 1996), when more than half of the islet tissue may be lost due to increased β-cell apoptosis and necrosis (Biarnes *et al.* 2002). A better understanding of the mechanisms leading to early death of transplanted islets could be used to improve the survival of transplanted beta cell mass, and to reduce the islet tissue required to achieve normoglycemia in diabetic recipients.

Early islet cell dysfunction and damage takes place before immunological rejection of the graft, and although it is more common in xenogeneic and allogeneic transplants it has been also described in syngeneic islet transplants (Biarnes *et al.* 2002, Kaufman *et al.* 1990), confirming the involvement of non-immunological processes. The mechanism of early graft failure is probably multifactorial, and includes technical problems during the transplantation process (Kaufman *et al.* 1988), inadequate mass of islet tissue (Montaña *et al.* 1993), hypoxia of islets (Dionne *et al.* 1993), the metabolic condition of the recipient (Merino *et al.* 1997), and the absence of survival factors present in the non-endocrine pancreas (Ilieva *et al.* 1999). In addition, non-specific inflammation at the grafted site, which may be partly related to damage to islets during isolation, could play a role on the initial fate of transplanted islets (Berney *et al.* 2001, Vargas *et al.* 1998). An increased expression of inflammatory cytokines may participate in this non-specific inflammation, and induce the functional stunning or destruction of transplanted islets (Berney *et al.* 2001, Gysemans *et al.* 2003, Osaza *et al.* 1997, Rabinovitch 1998).

The effects of pro-inflammatory cytokine interleukin-1β (IL-1β) in islet cells are particularly relevant (Mandrup-Poulsen 1996). IL-1β has been conclusively shown to impair glucose-stimulated insulin production in mouse, rat and human islets (Eizirik

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1991, Sandler *et al.* 1987, Giannoukakis *et al.* 2000), and to increase beta cell death (Delaney *et al.* 1997, Hoorens *et al.* 2001, Steer SA *et al.* 2006). *In vitro* observations indicate that the cytotoxic effect of IL-1β in islet cells involves the induction of nitric oxide synthase (iNOS) and the production of nitric oxide (NO) (Hoorens *et al.* 2001, Steer SA *et al.* 2006). In islet transplantation, it has been suggested that IL-1β may contribute to early graft dysfunction, but the analysis of IL-1β gene expression has been mostly limited to islets transplanted to NOD mice (Gysemans *et al.* 2000, 2003). Moreover, direct evidences of the presence of the cytokine in islet grafts have not yet been provided, and the question of the cellular origin of cytokines in the graft has not been investigated. In this study we provide evidence of the expression of both IL-1β and iNOS genes and proteins in the initial days after syngeneic islet transplantation.

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RESEARCH DESIGN AND METHODS

Experimental Design

Animals. Male Lewis rats (B&K Universal, U.K.), aged 7 to 10 wk, were used as donors and recipients of transplantation. The recipients were made diabetic by a single intraperitoneal injection of streptozotocin (STZ) (Streptozotocin, Sigma Immunochemicals, St Louis, MO, USA) 65 mg/kg body wt, freshly dissolved in citrate buffer (pH = 4.5). Diabetes was confirmed by the presence of hyperglycemia, weight loss, and polyuria and only those rats with blood glucose higher than 20 mmol/l on a minimum of two consecutive measurements were included in the study. Blood glucose was determined between 9 and 11 a.m. in non-fasting conditions, unless stated otherwise. Blood was obtained from the snipped tail and glucose was measured with a portable glucose meter (Glucocard, A. Menarini Diagnostics, Barcelona, Spain). Animals were kept under conventional conditions in climatized rooms with free access to tap water and standard pelleted food.

Insulin treatment. Twelve to 14 days after STZ injection, when diabetes was well established in injected rats, the animals were started on insulin in order to maintain blood glucose as close as possible to normal range on transplantation day and in subsequent days. This experimental approach was used to mimic human islet transplantation into diabetic patients, and to avoid the potentially confounding effects of hyperglycemia. Insulin was given as one subcutaneous implant of sustained insulin release (Linplant, Linshin Canada Inc., Scarborough, Canada) (Merino *et al.* 1997). Insulin treated groups were transplanted after 7 days on insulin and a minimum of 2 days of normoglycemia, and the insulin implants were removed the day of graft harvesting.

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Islet Isolation and Transplantation. Islets were isolated by collagenase (Collagenase P, Boehringer Mannheim Biochemicals, Mannheim, Germany) digestion of the pancreas as previously described (Montaña *et al.* 1993). All isolations were performed with the same batch of collagenase to avoid differences in endotoxin activity among isolations (Vargas *et al.* 1998). Isolated islets were hand-picked under a stereomicroscope two or three times until a population of pure islets was obtained. Islets >75 and <250 µm in diameter were counted into groups of 500 islets, and transplanted under the left kidney capsule of the recipient on the day of isolation. The islets were transplanted to normoglycemic, insulin-treated, STZ-diabetic rats or to normoglycemic non-STZ-injected rats. After transplantation, the capsulotomy was cauterized with a disposable low-temperature cautery pen (AB Medica, S.A, Barcelona, Spain), and the lumbar incision was sutured.

Graft harvesting. The kidney was exposed, and the kidney capsule surrounding the graft was incised and removed with the graft. The grafts were harvested 1, 3 or 7 days after transplantation and processed for RNA extraction and gene expression studies. For immunohistochemical analysis grafts harvested on days 1 and 3 after transplantation were immersed in 4% paraformaldehyde-phosphate buffered saline (PBS), and processed for paraffin embedding.

Gene Expression Studies

RNA isolation and complementary DNA (cDNA) synthesis. Total RNA was extracted from six groups of 500 freshly isolated islets and from day 1, 3 and 7 islet grafts using RNeasy Mini Kit (Qiagen, Crawley, U.K.), according to the manufacturer's instructions. Isolated islets and islet grafts were immersed in 350 µl guanidine isothiocyanate-containing buffer plus 3.5 µl β-mercaptoethanol. Samples were homogenized and lysed by mechanical disruption with repeated passing through a Pasteur pipette. The lysate was

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centrifuged and the non-lysed kidney capsule was discarded. Total RNA was eluted in diethyl pyrocarbonate (DEPC) treated water and quantified at 260 nm (DU® 640 Spectrophotometer, Beckman, Fullerton, USA).

To perform cDNA synthesis total RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) at 37ºC for 30 min and predenatured at 60ºC for 10 min with 500 ng random primers (Promega). cDNA synthesis was carried out on total RNA using Superscript reverse transcriptase (400U) (Invitrogen, USA) in a 40-µl reaction at 42ºC for 1 h in a Gene Amp PCR System 9600 (Perkin-Elmer, Norwalk, CT, USA).

PCR. PCR amplification was performed on 2–10 µl cDNA in a 50 µl reaction, containing 0.2 µM of each primer, 200 µM dNTPs and 1 U of Taq DNA polymerase recombinant (Invitrogen). The sequences of the specific oligonucleotide primer pairs used and the conditions for the PCR are given in Table 1. Samples were amplified by cycles of denaturation at 94ºC for 30 s., annealing at different temperature depending on the gene for 30 s., and elongation at 72ºC for 45 s in a Gene Amp PCR System 9600. Polymerase chain reaction cycles were 28 for cyclophilin and 34 for IL-1β and iNOS. The PCR products were electrophoresed on 2% agarose gels, transferred to nylon membranes and hybridized using oligonucleotide probes. The sequences of the oligonucleotide probes used and the temperature conditions of the hybridization are given in Table 2. Oligonucleotide probes were radiolabeled with ^{32}P-dATP, using T4 polynucleotide kinase. The hybridization procedure for oligonucleotide probes included prehybridization for 1 h in buffer containing 0.5 M phosphate buffer (pH 7.2), 10 mM EDTA, and 7% SDS, and overnight hybridization in the same buffer adding 20 pmol of the specific labeled probe. Post-hybridization washes were performed with 2× SSC/0.1% SDS at 37ºC and membranes were opposed to x-ray film.

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Semiquantitative method. To compare the relative expression of IL-1β and iNOS genes between different samples, we used a RT-PCR semiquantitative method. Reaction conditions were standardised for cycle number to observe linear amplification of the PCR products. The PCR for IL-1β and iNOS was performed with 34 cycles and for cyclophilin with 28 cycles, which were within the linear range of amplification. Under the cycles used, the PCR product signal was proportional to the amount of cDNA subjected to PCR amplification. We verified that the number of cycles used was in the exponential phase of amplification in each run collecting 15 µl of PCR product from all samples at 28, 30 and 34 cycles. PCR products were electrophoresed on 2% agarose gels and transferred to nylon membranes. The abundance of PCR products of interest was expressed in optical density (OD), normalized for the abundance of the cyclophilin signal amplified from the same cDNA sample. The values were expressed as percentage of the optical density of the interest gene relative to the coamplified internal control gene (cyclophilin gene). The percentage of the interest gene versus cyclophilin gene in graft samples was referred to the percentage obtained in a fresh islet preparation that was included in each PCR experiment.

Determination of kidney-specific gene. Islet grafts harvested with the kidney capsule can be contaminated with kidney cortex. To exclude the presence of kidney cortical cells in our RNA samples, we amplified the kidney-specific gene NKT, a gene product related to the organic ion transporter family (Vasir *et al.* 2001). The sequences of the specific oligonucleotide primer pairs used and the conditions for the PCR are given in Table 1. RT-PCR was performed as described above, and amplified through 40 cycles.

Immunohystochemical Studies.

Apoptosis detection. Three micrometer graft sections were double stained by immunoperoxidase for apoptotic nuclei using the TUNEL technique (In Situ Cell Death

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Detection Kit, ApopTag®, Intergen, Oxford, U.K.), and by alkaline phosphatase for the endocrine non-beta cells of the islets, as described (Biarnes *et al.* 2002). Beta cells and apoptotic nuclei were identified and counted using an Olympus BH-2 microscope connected to a video camera with a color monitor. When assessing apoptotic nuclei we excluded regions with necrosis. 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 when needed a second section was included.

Beta-cell apoptosis was also determined in 9 groups of 100 freshly islets isolated on different days and in 5 pancreases from control Lewis rats. Sections of isolated islets and pancreases were double stained and counted as described for grafts.

CD68, IL-1β, and iNOS immunohistochemistry. To identify the presence of macrophages we stained CD68-expressing cells (monocyte/macrophage lineage marker) in freshly isolated islets and in islet grafts. Rat spleen sections were used as a positive control. Endogenous peroxidases were blocked with 1% hydrogen peroxidase solution and antigen retrieval was performed by incubation in citrate buffer. Then, sections were blocked with 5% horse serum (Biological Industries, Beit Haemek, Israel), and incubated overnight at 4ºC with mouse anti-rat CD68 antibody (final dilution 1:100) (Serotec, Oxford, U.K.). Immunostaining for CD68 was performed using an Immuno Pure® ABC Peroxidase Mouse IgG Staining Kit (Pierce, Rockford, USA) according to the manufacturer's instructions. Peroxidase reaction was visualized with 3,3'-diaminobenzidine tetrahydrochloride and hydrogen peroxide. Bright-field microscope was used to visualize the CD68-staining sections.

To identify the presence of IL-1β and iNOS proteins and the contribution of macrophages to their expression, sections were incubated overnight at 4ºC with goat

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anti-rat IL-1β antibody (final dilution 1:30) (R&D, Mckinley Place, MN, USA), or goat anti-mouse and rat NOS2 (iNOS) antibody (final dilution 1:10) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and with mouse anti-rat CD68 antibody (final dilution 1:100) (Serotec) primary antibodies. To minimize autofluorescence, sections were stained with Sudan Black B (Sigma) for 30 min. Sections were then incubated with donkey FITC-conjugated monoclonal anti-goat secondary antibody (final dilution 1:400) (Abcam, Cambridge, UK) and with rabbit anti-mouse AlexaFluor 546-conjugated secondary antibody (final dilution 1:400) (Dako). Incubation with DRAQ5™ (Biostatus Limited, Leicestershire, UK) was used to dye cell nuclei with a low infra-red fluorochrome. A Leica TC6-SL Spectral confocal microscope was used to visualize the fluorescence and images were processed with Leica Confocal Software, version 2.5 (Leica Microsystems Heidelberg GmbH, Mannheim, Germany).

Islets treated with 50 ng/ml TNF-α + 750 U/ml IFN-γ + 10 μg/ml LPS for 4 h were used as positive control for IL-1β-expressing cells. Islets treated with 50 U/ml IL-1β for 24 h were used as positive control for iNOS-expressing cells.

Statistical Analysis. Results were expressed as mean and standard error of the mean (mean ± SEM). Differences between means were evaluated by one-way analysis of variance (ANOVA). The Fisher's PLSD (protected least significant difference) method was used to determine specific differences between means when determined as significant by ANOVA main effects analysis. A p value of less than 0.05 was considered significant.

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RESULTS

Metabolic evolution. STZ-treated rats developed severe hyperglycemia with polyuria and weight loss shortly after STZ injection. Hyperglycemia was corrected with insulin treatment, and after transplantation the animals were even moderately hypoglycemic (Table 3). Severe hyperglycemia (>20 mmol/l) recurred in all rats when the graft and insulin implants were harvested. Transplantation did not modify blood glucose in non-diabetic, non-STZ injected rats.

Gene expression of IL-1β and iNOS in freshly isolated islets and in islet grafts. IL-1β mRNA was detected in islets after isolation in 5 of 6 samples from different isolation procedures, and was up-regulated after transplantation. IL-1β mRNA was 9-fold increased on day 1 after transplantation, it was still 7-fold increased on day 3 compared with fresh islets, and declined towards pre-transplantation levels on day 7 (Fig. 1). iNOS mRNA showed a similar pattern of expression than IL-1β: on days 1 and 3 after transplantation it was 14- and 4-fold higher respectively than in freshly isolated islets. IL-1β and iNOS mRNA levels in individual islet grafts were significantly and positively correlated ($r = 0.56$, $p < 0.05$). To exclude the presence of kidney cortex contaminating the islet graft RNA samples, we examined the expression of NKT mRNA. No expression of NKT mRNA was detected in 7 day-islet grafts (data not shown).

Since insulin-treated rats were often hypoglycemic after transplantation, we studied an additional group of normoglycemic rats, not injected with STZ ($n = 3$), that was transplanted with 500 islets to determine whether hypoglycemia could play a role in the higher IL-1β and iNOS mRNA expression. In these strictly normoglycemic rats, IL-1β and iNOS expression on day 1 after transplantation was 12- and 9-fold higher respectively than in freshly isolated islets, indicating that hypoglycemia was not

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responsible for the increased IL-1β and iNOS expression in the insulin-treated diabetic groups (Fig. 1).

β-cell death. β-cell apoptosis was similar in freshly isolated islets immediately after islet isolation ($0.09 \pm 0.04\%$) and control pancreases ($0.04 \pm 0.01\%$), but was significantly increased on day 3 grafts ($0.30 \pm 0.01\%$, ANOVA $p < 0.001$, Fisher PLSD, $p < 0.01$ between transplanted islets, and pancreas and isolated islets) (Fig. 2). In addition, extensive areas of necrosis, similar to those previously reported in syngeneic mice islet grafts (Biarnes *et al.* 2002) were identified.

CD68-, IL-1β- and iNOS-positive cells in islet grafts. In islet grafts, macrophages were abundant in the periphery of the islet tissue and in the necrotic areas (Fig. 3). In contrast, few CD68-positive cells were detected within the islet tissue.

The expression of IL-1β and iNOS proteins was investigated by inmunocytochemistry in islet grafts transplanted to normoglycemic recipients and harvested on days 1 (n = 3) and 3 (n = 3) after transplantation. We detected the presence of IL-1β and iNOS positive cells in all islet grafts, with no significant differences between days 1 and 3 in number or distribution of positive cells.

To determine the role of macrophages in IL-1β and iNOS expression, we double stained the graft sections with anti-CD68 antibody, and anti-IL-1β or anti-iNOS antibodies. The double-stained sections were analyzed by confocal microscopy (Fig. 4). IL-1β expression was detected almost exclusively in CD68-positive cells that accounted for ~95% of IL-1β-expressing cells on days 1 and 3 after transplantation. 10% of CD68-positive cells expressed IL-1β on day 1, and 14% on day 3. The rare IL-1β-positive/CD68-negative cells (~5% of all IL-1β-positive cells) were all found within the islets.

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iNOS expression was also predominantly detected in CD68-positive cells that accounted for 88% and 77% of iNOS positive cells on days 1 and 3 respectively. 34% of CD68-positive cells expressed iNOS on day 1, and 8% on day 3. Compared to the rare expression of IL-1β in cells that did not express the monocyte/macrophage lineage marker, iNOS expression was more common in CD68-negative cells. These iNOS-positive/CD68-negative cells (12% and 23% of iNOS positive cells on days 1 and 3 after transplantation) were found both inside and outside the islet tissue.

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DISCUSSION

In this study we show that the expression of IL-1β and iNOS genes was already detectable in freshly isolated islets and it increased significantly after transplantation. The expression of IL-1β and iNOS genes was maximal on day 1 after transplantation and then declined progressively. In addition, IL-1β- and iNOS-positive cells were found in islet grafts, and macrophages were identified as the main cellular source of both proteins. The results confirm the presence of an inflammatory process in early islet transplantation, that takes place even in syngeneic transplantation, and suggest that IL-1β plays a role in the extensive beta cell death found in the initial days after islet transplantation.

Similar to our previous results in mice islet grafts (Biarnes *et al.* 2002), we found that rat islet grafts show necrosis and increased apoptosis shortly after transplantation. Non-specific inflammation at the grafted site may contribute to this initial islet damage which results in massive loss of transplanted tissue (Biarnes *et al.* 2002), and can lead to primary non graft function. Ozasa *et al.* (1997) found increased levels of IL-1α, IL-2, IL-6 and IFN-γ transcripts in syngeneic and allogeneic islet grafts compared with freshly isolated islets. However, they could not perform a quantitative comparison of the expression of transcripts in different days, and they did not analyze the presence of the relevant IL-1β transcript. IL-1β is a candidate to contribute to early graft inflammation in islet transplantation, but its expression has been investigated almost exclusively in autoimmune models in islet syngeneically transplantated to NOD mice (Gysemans *et al.* 2000, 2003). Cardozo *et al.* (2003) detected the expression of IL-1β gene in Balb/c islet isografts eight hours after transplantation, but isolated, non-transplanted, islets were not studied and it could not be established whether IL-1β expression was increased in islet grafts compared to isolated islets. This is an important question, because previous studies have shown that the endotoxin activity present in collagenase preparations used in islet

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isolation elicits an inflammatory cytokine response in macrophages, and may be responsible for the induction of several pro-inflammatory cytokine genes in islets during the isolation process (Vargas *et al.* 1998, Berney *et al.* 2001). In this study, we have found clearly increased levels of IL-1β mRNA in islet grafts on day 1 after transplantation compared to isolated islets and to days 3 and 7 after transplantation, indicating that the expression of IL-1β was enhanced by the transplantation process.

This is the first study that directly shows the presence of IL-1β and iNOS proteins in recently transplanted islet grafts. IL-1β is the major regulator of iNOS expression in beta cells (Eizirik *et al.* 1992, Karlsen *et al.* 1995), and in rat islets IL-1β alone is sufficient to stimulate iNOS expression, and nitric oxide (NO) production. NO has a fundamental role in cytokine-induced beta cell damage (Eizirik *et al.* 1992, Karlsen *et al.* 1995, Liu *et al.* 2000, Steer *et al.* 2006). Thus, the expression of iNOS suggests that the presence of IL-1β in the graft was biologically significant. The increased expression of IL-1β and iNOS genes, along with the identification of IL-1β and iNOS proteins in grafts, confirms the presence of an early inflammatory process in islet transplantation, and strongly suggests that they are important mediators of graft inflammation and islet damage in early islet transplantation. The relevance of this inflammatory process may go beyond the induction of direct islet damage in accordance with the proposed role of inflammation trigger of the immune response (Matzinger 2002). Thus, in islet allotransplantation the initial non-specific inflammation could enhance the subsequent immune rejection.

The question of the cellular origin of pro-inflammatory cytokines in islet grafts had not been previously investigated. We did not detect the kidney-specific gene NKT in graft homogenates, excluding the contamination from kidney tissue and leaving the graft as the only source of IL-1β and iNOS mRNA. Using confocal microscopy we found that macrophages were, by large, the main source of IL-1β and iNOS in islet grafts. The role of

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macrophages in early graft failure has been previously suggested by reports of improved graft survival with treatments that depleted or inactivated macrophages (Kaufman *et al.* 1990, 1994, Kenmochi *et al.* 1996, Bottino *et al.* 1998).

Other cell types, in addition to macrophages, had a less important contribution to IL-1β and iNOS expression in islet grafts, as indicated by the 5% of IL-1β-positive cells and 10–25% of iNOS-positive cells that were CD68-negative. IL-1β expression by cells other than macrophages was found only within the islets. Transplanted islet preparations contain, in addition to the endocrine cells of the islets, exocrine tissue, capillary endothelial cells, macrophages, dendritic-like cells, fibroblasts and other minor cell populations. Ductal and endothelial cells were source of IL-1β in human islets in response to LPS + TNF-α + IFN-γ, although they represented a small number when compared with macrophages (Matsuda *et al.* 2005). In contrast, beta cells were the major source of NO in rat and human islets in response to IL-1β or IL-1β + IFN-γ respectively (Corbett *et al.* 1995, Arnush *et al.* 1998). We found that iNOS-positive/CD68-negative cells were similarly distributed inside and outside the islets. The intra-islet expression of iNOS by cells other than macrophages is important because it has been hypothesized that NO must be produced by beta cells to induce beta cell damage (Thomas *et al.* 2002). Nevertheless, the expression of iNOS by the abundant macrophages infiltrating the graft probably contributed also to beta cell damage, similarly to what has been described in other tissues (Bruns *et al.* 2000).

In summary, the increased IL-1β and iNOS gene and protein expression in syngeneic islet grafts confirms the presence of a non-specific inflammatory response in the initial days after islet transplantation. Macrophages were the main cellular source of IL-1β and iNOS, confirming their essential role in early graft inflammation. Considering the well

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established cytotoxicity of IL-1β and NO, they probably contribute to the initial massive loss of transplanted islet cells and to the phenomenon of early graft failure.

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FIGURE LEGENDS

Figure 1. Expression of IL-1β gene (A) and iNOS (B) in freshly isolated islets, in syngeneic islet grafts transplanted to normoglycemic, insulin-treated, STZ-diabetic rats studied on days 1, 3 and 7 after transplantation (open bars), and in syngeneic islet grafts transplanted to normoglycemic, non-STZ treated, rats studied on day 1 after transplantation (grey bar). Values are means ± SEM. ANOVA, $p < 0.05$, Fisher PLSD, $*p < 0.01$ between IL-1β expression in fresh islets and day 1 after transplantation; $*p < 0.05$ among iNOS expression in day 1 after transplantation and all other groups.

Figure 2. A. Beta cell apoptosis in normal pancreas, freshly isolated islets and day 3 grafts. Values are means ± SEM. ANOVA, $p < 0.005$; Fisher PLSD, $* p < 0.01$ compared to other groups. B. Apoptosis in day 3 transplanted β-cell (arrow). Apoptotic nuclei were stained using the TUNEL technique and visulised with 3,3'diaminobenzidine tetrahydrochloride, and non-β-cells were stained with a cocktail of antibodies anti-glucagon, anti-somatostatin, and anti-pancreatic polypeptide, and detected by alkaline phosphatase.

Figure 3. Macrophage detection in islet grafts. Sections were stained using an anti-CD68 antibody (monocyte/macrophage lineage marker), and visualized with 3,3'-diaminobenzidine tetrahydrochloride on bright field microscope. A typical day 3 graft is shown. Aboundant macrophages were found in the periphery of islet tissue and in necrotic areas.

Figure 4. Immunofluorescence detection of IL-1β- and iNOS postive cells in islet grafts. Sections were double stained for IL-1β or iNOS, and CD68. IL-1β expression

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colocalized mainly with CD68-positive cells (arrows) (A), but scarce intraislet IL-1β-positive/CD68 negative cells were also found (B). iNOS expression colocalized mainly with CD68-positive cells (arrows) (C), but less abundant iNOS-positive/CD68-negative cells were also found (D). "I" indicates islet tissue.

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Table 1. Gene specific primers for PCR

Gene name	Primer sequence 5'-3'	Annealing (°C)	$MgCl_2$ (mM)	Size (bp)
IL-1β	5' dCCTGTGGCCTTGGGCCTCAA	60	2	204 bp
	3' dGGTGCTGATGTACCAGTTGGG			
iNOS	5' dTCCCCCACATTCTCTTTCCTTT	58	1.5	204 bp
	3' dCAAGCGGTCGTTGGGAGTG			
NKT	5' dATCTTGCCTCCGCTTTCC	55	1.4	287 bp
	3' dAGGTTCCCGACACAGCTG			
Cyclophilin	5' dAACCCCACCGTGTTCTTC	55–60	1.4–2	399 bp
	3' dTGCCTTCTTTCACCTTCCC			

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Table 2. Oligonucleotide probes for hybridization

Probes	Oligo probe sequence 5'-3'	Hybridization (°C)
IL-1β	5' dATTGTTTGGGATCCACACTCTCCAG	65
iNOS	5' dCGGGATGGCGCCTCCTG	58
Cyclophilin	5' dTTTCTCTCCGTAGATGGACTTGCCACCAGTGCCA	63

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Table 3. Characteristics of experimental groups

Treatment	Day of graft removal	*n*	First Day of Insulin Treatment		Transplantation Day		Day Graft Removal	
			Blood glucose (mmol/l)	Body weight (g)	Blood glucose (mmol/l)	Body weight (g)	Blood glucose (mmol/l)	Body weight (g)
Tx+STZ-injected +insulin-treated	1	5	28.1±1.8	171±5	3.3±0.4	218±12	2.3±0.3	217±12
Tx+STZ-injected +insulin-treated	3	9	27.5±1.7	171±5	3.5±0.5	227±6	3.1±0.3	233±6
Tx+STZ-injected +insulin-treated	7	4	28.0±1.1	167±7	3.9±1.0	206±7	2.5±0.2	236±5
Tx, non-STZ	1	6	—	—	5.4±0.2	224±8	5.2±0.2	213±7
Tx, non-STZ	3	3	—	—	5.3±0.2	221±17	5.5±0.5	221±15
Control *	—	7	5.7±0.1	167±6	6.0±0.3	200±3	5.7±0.2	233±5

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

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Fig 1.

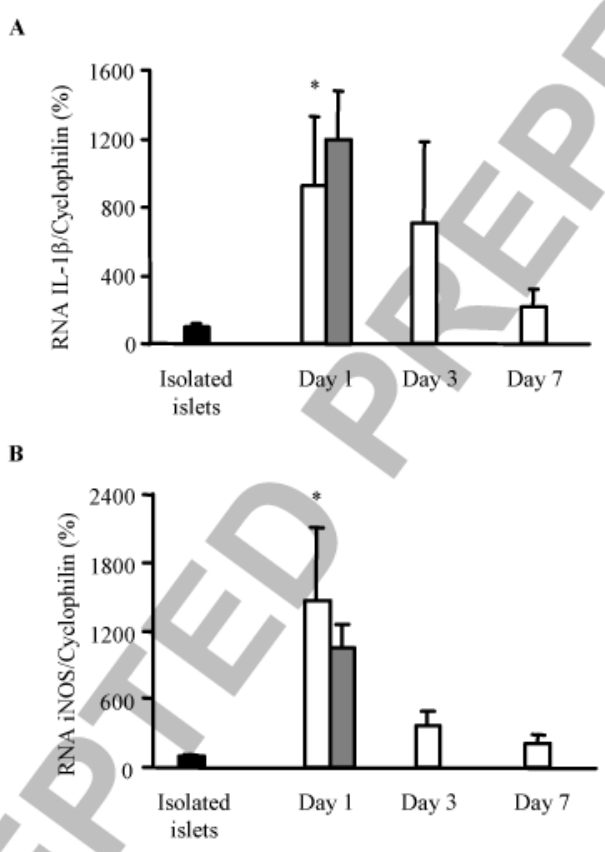

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Fig 2.

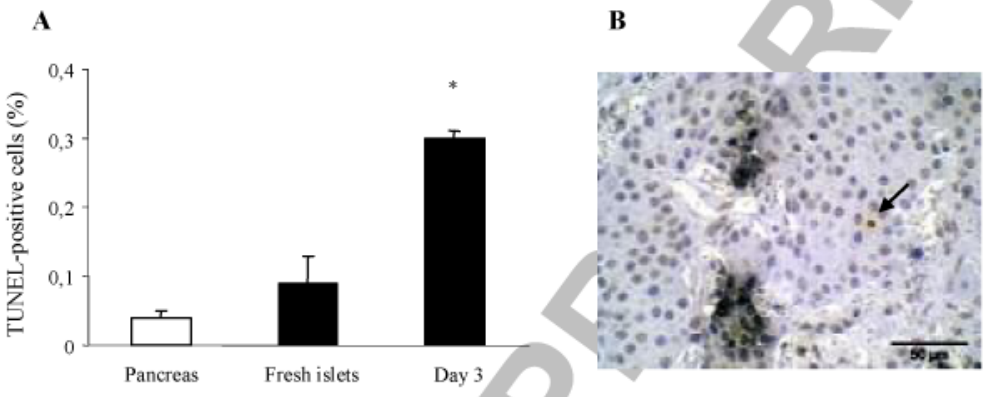

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Fig. 3

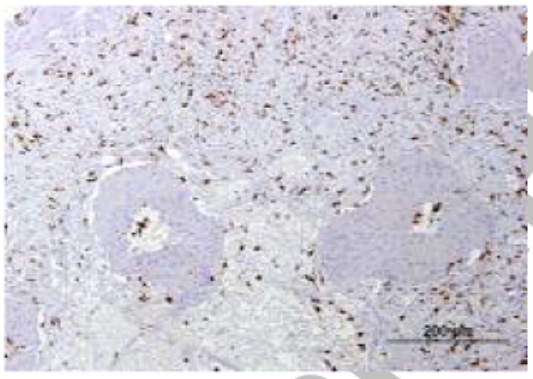

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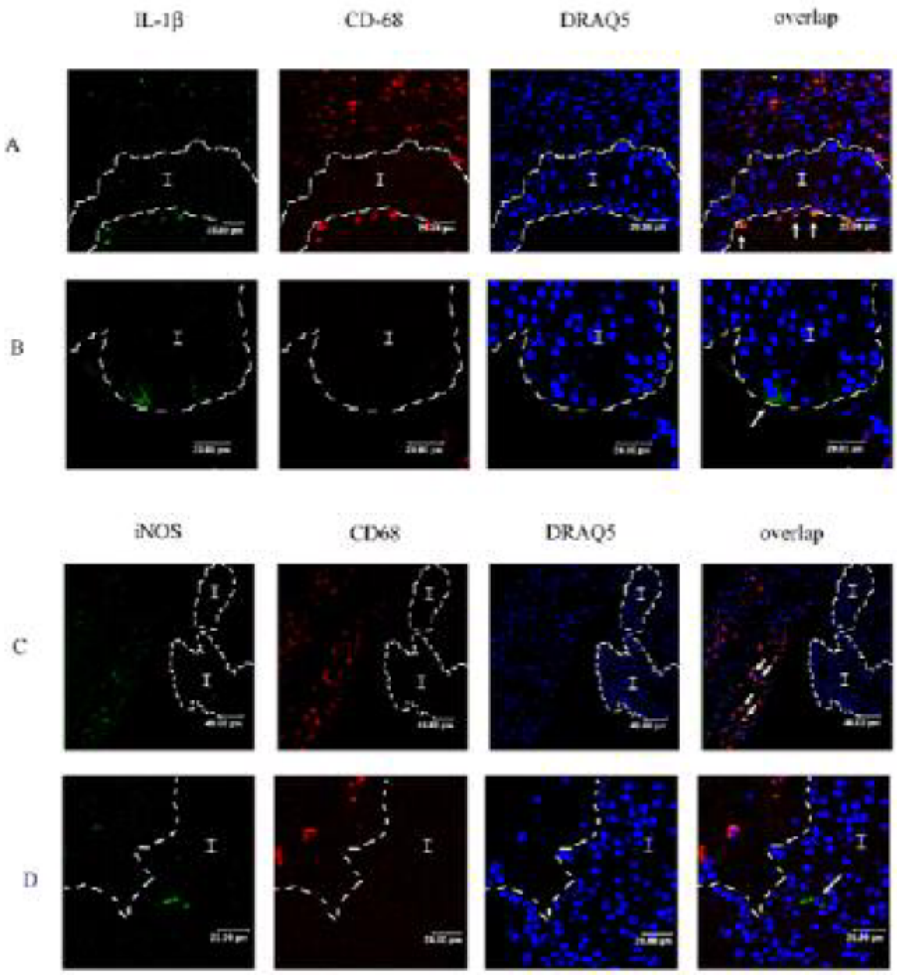
Fig. 4.



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CELL TRANSPLANTATION

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RE: Manuscript No. CT-1530

Dear Dr. Montanya:

I am pleased to inform you that your manuscript, "Role of Blood Glucose in Cytokine Gene Expression in Early Syngeneic Islet Transplantation" **has been accepted for publication in *Cell Transplantation: The Regenerative Medicine Journal*.**

Please mail two copies of your manuscript with two sets of figures on glossy paper (with the figure number on the reverse side) along with a new IBM-formatted disk (or CD) to Yanet Florez, Diabetes Research Institute R-134, 1450 NW 10th Avenue, Miami, FL 33136.
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Enclosure

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(With manuscript and disk)

Role of blood glucose in cytokine gene expression in early syngeneic islet transplantation

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Running title: Cytokine expression in islet grafts

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ABSTRACT

In islet transplantation, local production of cytokines at the grafted site may contribute to the initial non-specific inflammation response. We have determined whether the metabolic condition of the recipient modulates the cytokine expression in islet grafts in the initial days after transplantation. Normoglycemic and hyperglycemic streptozotocin-diabetic Lewis rats were transplanted with 500 syngeneic islets, an insufficient beta cell mass to restore normoglycemia in hyperglycemic recipients. The expression of IL-1β, TNF-α, IFN-γ, IL-6, IL-10, and IL-4 genes was determined by real-time PCR in freshly isolated islets, in 24 h-cultured islets and in islet grafts on days 1, 3 and 7 after transplantation. IL-1β mRNA was strongly and similarly increased in normoglycemic and hyperglycemic groups on days 1, 3, and 7 after transplantation compared with freshly isolated and cultured islets. TNF-α mRNA was also strongly increased on day 1, and it remained increased on days 3 and 7. IL-6 and IL-10 were not detected in freshly isolated islets, but their expression was clearly enhanced in 24 h-cultured islets and islet grafts. IL-6 was further increased in hyperglycemic grafts. IL-10 expression was increased in both normoglycemic and hyperglycemic grafts on day 1 after transplantation, and remained increased in hyperglycemic grafts compared to 24 h-cultured islets. IFN-γ mRNA was barely detected in a few grafts, and IL-4 mRNA was never detected. Thus, the inflammatory response in islet grafts was maximal on day 1 after transplantation, it was sustained, although at lower levels, on days 3 and 7, and it was partly enhanced by hyperglycemia.

Key words: islet transplantation, cytokine, real-time PCR, hyperglycemia

INTRODUCTION

The promising results achieved by islet transplantation in the treatment of type 1 diabetes (35) are attenuated by the severe limitation imposed by the scarcity of islet tissue available for transplantation and the high number of islets required for successful transplantation (35). The enthusiasm generated by the Edmonton protocol has been recently tempered by the demonstration of high recurrence of diabetes (34). The vulnerability of islets in the initial days after transplantation, when more than 60% of the islet tissue is lost (4), clearly increases the number of islets required to achieve initial normoglycemia after transplantation, and may play also a role in the long term failure of the graft (15). Reduction of this initial beta cell death has been shown to improve the metabolic outcome of the graft (25). The study of candidate genes to contribute to initial damage of transplanted islets could improve our understanding of the mechanisms leading to early damage of transplanted islets and could be used to design strategies aimed to improve the survival of transplanted islets.

The mechanism of early graft failure is probably multifactorial, and although mediators of this initial beta cell death are not well known, it is generally accepted that after transplantation an inflammatory response takes place in the context of a "transplant environment activation" comprising ischemia/reperfusion damage and production of pro-inflammatory mediators (32). This inflammatory response occurs before immunological rejection or recurrence of autoimmunity take place, and is a general phenomenon afflicting syngeneic, allogeneic and xenogeneic islet transplantation (32). Although inflammatory cytokines have been pointed out as important players in this non-specific inflammation (32), their expression has been rarely determined in islet grafts in this context.

It is clearly established that the metabolic condition of the recipient modifies the outcome of islet transplantation. Islet grafts exposed to sustained hyperglycemia show impaired beta cell function (14,16,43), limited replicative capacity, increased beta cell apoptosis (4), and reduced beta cell mass (11,24). Moreover, we have reported that insulin-induced normoglycemia has a beneficial effect on the outcome of pancreatic islets transplanted to diabetic recipients in rodents (23). It is not known, however, whether the deleterious effects of hyperglycemia on transplanted islets enhance the non-specific inflammation characteristic of early islet transplantation. Thus, the aim of the study was to determine the expression of pro- and anti-inflammatory cytokine genes in the initial days after syngeneic islet transplantation and to study whether blood glucose modulates their expression in islet grafts.

MATERIALS AND METHODS

Animals

Male Lewis rats (Harlan, Horst, The Netherlands) aged 7 to 10 wk, were used as donors and recipients of transplantation. Hyperglycemic recipients were made diabetic by a single intraperitoneal injection of streptozotocin (STZ) (Streptozotocin, Sigma Immunochemicals, St Louis, MO, USA) 65 mg/kg body wt, freshly dissolved in citrate buffer (pH = 4.5). Only those rats with a blood glucose higher than 20 mmol/l on two consecutive measurements were transplanted. Blood glucose was determined between 9 and 11 a.m. in non-fasting conditions. Blood was obtained from the snipped tail and glucose was measured with a portable glucose meter (Glucocard, 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 Design

Normoglycemic, non-STZ-injected, and hyperglycemic, STZ-injected, Lewis rats were transplanted with 500 freshly isolated syngeneic islets. Since 500 syngeneic islets are an insufficient beta cell mass to consistently restore normoglycemia in this model (40), the diabetic animals were expected to remain hyperglycemic after transplantation. Grafts from normoglycemic and hyperglycemic rats were harvested on days 1, 3 or 7 after transplantation (six groups, n = 6 for each experimental group).

Six groups of 500 freshly isolated islets were used to determine cytokine expression in islets before transplantation. In addition, six groups of 500 islets were cultured for 24 h in RPMI-1640 medium supplemented with 11.1 mM glucose and 10% Fetal Calf Serum (FCS, Biological Industries, Beit Haemek, Israel) at 37ºC and 5% CO_2 to determine cytokine expression after culture.

Islet isolation, transplantation, and graft harvesting

Islets were isolated by collagenase (Collagenase P, Boehringer Mannheim Biochemicals, Germany) digestion of the pancreas as previously described (27). All isolations were performed with the same batch of collagenase to avoid differences in endotoxin activity among isolations (41). Isolated islets were hand-picked under a stereomicroscope two or three times until a population of pure islets was obtained. Islets 75–250 μm diameter were counted into groups of 500 islets and transplanted into the recipient on the same day of the isolation. Animals were anesthetized with a mixture of ketamine (100 mg/kg), diazepam (7.5 mg/kg) and atropine (0.05 mg/kg) and the left kidney was exposed through a lumbar incision. A capsulotomy was performed and islets were injected under kidney capsule. After transplantation, the capsulotomy was cauterized with a disposable low-temperature cautery pen (AB Medica, S.A, Barcelona, Spain), and the lumbar incision was sutured. To harvest the grafts, the kidney was exposed, and the kidney capsule surrounding the graft was incised and removed with the graft. After removal from the kidney, the graft was immediately immersed in lysis buffer and total RNA was extracted.

RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was extracted from freshly isolated islets, 24 h-cultured islets, and islet grafts using RNeasy Mini Kit (Quiagen, Crawley, UK), according to the manufacturers protocol. Islets and grafts were immersed in lysis buffer containing 350 μl guanidine isothiocyanate-containing buffer plus 3.5 μl β-mercaptoethanol. Samples were homogenizated and lysed by mechanical disruption with repeated passing through a Pasteur pipette. The lysate was centrifuged and the non-lysed kidney capsule was discarded. Total RNA was eluted in RNase-free water, quantified at 260 nm using DU® 640 Spectrophotometer (Beckman, Fullerton, USA) and its integrity visualized in 1% agarose gel.

A constant amount of 1 μg of total RNA was treated with 1 U RQ1 RNase-free DNase (Promega, Madison, USA) at 37ºC for 30 min and then predenatured at 65ºC for 10 min with 500 ng random hexamer primers (Promega,) and 10 mM of dNTP mix (10 mM each dATP, dGTP, dCTP, and dTTP) (Invitrogen, Carlsbad, USA). cDNA synthesis was carried out in a final volume of 40 μl mixture containing 5× First strand buffer (Invitrogen), 40 U RNasin (Promega), 0.1 M DTT and 400 U SuperScript™ II RNase H^- reverse transcriptase (Invitrogen). Reactions were performed in a GeneAmp PCR System 9600 Perkin-Elmer Thermocycler (Applied Biosystems, Cheshire, United Kingdom) at the following conditions: 25ºC for 10 min, 42ºC for 50 min, and stopped at 70ºC for 15 min. Resulting cDNA was stored at –20ºC until use.

Relative quantification gene expression using real-time PCR

cDNA of IL-1β, TNF-α, IFN-γ, IL-6, IL-10, and IL-4 were amplified and quantified by real-time PCR (ABI Prism® 7700, Applied Biosystems). Cytokine expression levels were normalized using eukaryotic rRNA 18S expression as internal control (33). Gene expression signals (C_T, or threshold cycle) were used to calculate $\Delta C_T = C_{T,\text{ target gene}} - C_{T,\text{ 18S}}$ (47). Since ΔC_T can be used when the amplifying efficiencies of both amplicons (target gene and internal control) are similar, we established the similarity of PCR amplification efficiencies of target gene and internal control for every pair of amplicons. With the exception of IL-4, that could not be detected, we confirmed that the absolute value of the slope of log input amount versus the difference between C_T for target gene and C_T for reference gene was closer to zero for all target genes (User Bulletin #2, ABI Prism 7700 Sequence Detection System, Applied Biosystems).

The target and the internal control genes were amplified in separated wells. For every PCR reaction, 1 μl of each cDNA sample (corresponding to 25 ng of starting RNA for target gene, and 25 pg for the internal control 18S) were mixed with 2× TaqMan®

Universal PCR Master Mix (12.5 μl), 20× Target or Internal control Primers and Probe (PDAR, TaqMan® Pre-Developed Assay Reagents, Applied Biosystems) (1.25 μl) in a total volume of 25 μl. Amplification was performed following the universal amplification program: 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 seconds, and 60ºC for 1 minute. Data were analyzed using the SDS software (Applied Biosystems).

CD68 immunohistochemistry. To identify the presence of macrophages, a potential source of pro-inflammatory cytokines in islet grafts, we stained CD68-expressing cells (monocyte/macrophage lineage marker) in freshly isolated islets and in islet grafts. Rat spleen sections were used as a positive control. Endogenous peroxidases were blocked with 1% hydrogen peroxidase solution and antigen retrieval was performed by incubation in citrate buffer. Then, sections were blocked with 5% horse serum (Biological Industries, Beit Haemek, Israel), and incubated overnight at 4ºC with mouse anti-rat CD68 antibody (final dilution 1:100) (Serotec, Oxford, U.K.). Immunostaining was performed using an Immuno Pure® ABC Peroxidase Mouse IgG Staining Kit (Pierce, Rockford, USA) according to the manufacturer's instructions. Peroxidase reaction was visualized with 3,3'-diaminobenzidine tetrahydrochloride and hydrogen peroxide. Bright-field microscope was used to visualize the CD68-staining sections.

Statistical Analysis

Results were expressed as mean and standard error of the mean (mean ± SEM). Differences among groups were evaluated using the one-way analysis of variance (ANOVA) or the Kruskal-Wallis *H* test, and when determined as significant (*p* value of less than 0.05) the Fisher's PLSD (protected least significant difference) method, or the Mann-Whitney *U* test was used to determine specific differences between groups. Subsequently the Hochberg's Sequential Method was used as post hoc test to adjust the Type I error (α) for multiple testing. However, the use of adjustments for multiple tests

has been seriously questioned (29), and it increases the likelihood of type II errors. Thus, the result of the Mann-Whitney *U* test is presented when it is statistically significant and it is followed by the result after the adjustment with the Hochberg's Sequential Method. The statistical software SPSS 12.0 and the Multiplicity Program from the Department of Biomathematics of The University of Texas M.D, Anderson Cancer Center (Houston, TX) were used.

RESULTS

Metabolic evolution

Blood glucose and body weight of experimental groups are summarized in Table 1. On transplantation day, blood glucose level was 28.3 ± 1.1 mmol/l in hyperglycemic group and 6.1 ± 0.2 mmol/l in normoglycemic group. STZ-diabetic rats remained hyperglycemic during the study confirming that 500 islets are an insufficient mass to restore normoglycemia.

Cytokine mRNA Expression

The total amount of RNA obtained from 500 isolated islets was 9.9 ± 1.0 μg, and 24 h-cultured islets had a similar total RNA content (11.0 ± 0.8 μg). The RNA recovered from the 500-islet grafts was reduced to ≈ 40% of the RNA obtained from 500 freshly isolated islets or from 500 cultured islets. This reduction is in agreement with the previously reported reduction in beta cell mass in the initial days after islet transplantation (4). There were no differences in recovered total RNA among days after transplantation or between normoglycemic and hyperglycemic groups (Fig. 1).

In freshly isolated islets IL-1β and TNF-α mRNA were detected at low levels, whereas IL-6 and IL-10 transcripts were barely detectable, and IFN-γ and IL-4 mRNA were not detected (Fig. 2). The expression of IL-6 and IL-10 genes became clearly detectable in 24 h-cultured islets. The expression of IL-1β, TNF-α, IL-6 and IL-10 was increased in islet grafts compared to freshly isolated islets. In contrast, IFN-γ mRNA remained undetectable in cultured islets and it was barely detectable or undetectable in islet grafts, whereas IL-4 was never detected in cultured islets or islet grafts. Thus, the detailed analysis of the expression of cytokines was limited to those consistently detected, IL-1β, TNF-α, IL-6 and IL-10.

IL-1β. IL-1β mRNA was detected in freshly isolated islets and in 24 h-cultured islets. On day 1 after transplantation, IL-1β expression increased strongly in both normoglycemic and hyperglycemic groups compared with freshly isolated ($p = 0.002$) and cultured ($p = 0.002$) islets. Although IL-1β expression decreased on days 3 and 7 after transplantation compared to 1 day grafts, it remained higher than in freshly isolated and cultured islets even when adjusted for multiple testing. Hyperglycemia did not modify IL-1β expression at any time after transplantation.

TNF-α. TNF-α mRNA was detected in freshly isolated islets and in 24 h-cultured islets. TNF-α expression increased on day 1 after transplantation both in normoglycemic and hyperglycemic groups, and although it decreased on days 3 and 7, it remained higher than in freshly isolated ($p = 0.002$) and 24 h-cultured islets ($p = 0.002$). On day 1 after transplantation TNF-α gene expression was higher in grafts from hyperglycemic recipients ($p = 0.026$), but the difference was not statistically significant when it was adjusted for multiple testing. On days 3 and 7 grafts transplanted to normoglycemic and hyperglycemic recipients showed comparable levels of TNF-α transcripts.

IL-6. IL-6 mRNA was barely detectable and could not be quantified in freshly isolated islets, but it was clearly detected in 24 h-cultured islets, and was further increased on day 1 after transplantation in islet grafts exposed to hyperglycemia ($p = 0.009$) a difference that was in the limit of significance after adjusting for multiple testing ($p = 0.054$). On days 3 and 7 after transplantation, IL-6 gene expression in hyperglycemic groups remained increased to values similar to those of 24 h-cultured islets. In contrast, in normoglycemic groups IL-6 was not increased on day 1 compared to 24 h-cultured islets, and was even reduced on days 3 ($p = 0.015$), and 7 ($p = 0.015$), a

difference that was of borderline significance after adjusting for multiple testing ($p = 0.060$).

IL-10. IL-10 mRNA was barely detectable and could not quantified in freshly isolated islets, but it was clearly detected in 24 h-cultured islets, and was further increased on day 1 after transplantation in both normoglycemic ($p = 0.002$) and hyperglycemic ($p = 0.002$) grafts, and it remained increased in hyperglycemic grafts on day 3 ($p = 0.009$). These differences remained statistically significant when adjusted for multiple testing. Although IL-10 gene expression was also higher on day 7 in hyperglycemic grafts ($p = 0.026$) the difference was not statistically significant when it was adjusted for multiple testing. In contrast, in normoglycemic groups IL-10 gene expression was not increased on days 3 and 7 after transplantation compared to 24 h-cultured islets.

CD68-positive cells in islet grafts. In islet grafts, macrophages were abundant in the periphery of the islet tissue and in the necrotic areas that are commonly found (3) in the initial days after transplantation (Fig. 3). A few CD68-positive cells were identified in freshly isolated islets, in agreement with previously published data (1).

DISCUSSION

We have identified the expression of pro-inflammatory cytokines IL-1β, TNF-α, IL-6, and anti-inflammatory IL-10 in syngeneic islet grafts immediately after transplantation. In contrast, the expression of IFN-γ and IL-4 genes was barely detectable or undetectable. Cytokine gene expression in islet grafts was maximal on day 1 after transplantation, and it declined thereafter even though in most cases it remained higher than in freshly isolated islets or 24 h-cultured islets. This inflammatory response was enhanced in islets transplanted to hyperglycemic recipients.

Previous studies have shown that the endotoxin activity present in collagenase preparations elicits an inflammatory response that may be responsible for the induction of several pro-inflammatory cytokine genes in islets during the isolation process (41). Thus, the cytokine expression found in the initial days after transplantation, and particularly on day 1, could reflect the inflammation induced by the isolation procedure. To minimize differences due to endotoxin-induced inflammation, all isolations were performed with the same batch of collagenase. Moreover, in order to ascertain the contribution of the isolation and transplantation procedures to cytokine gene expression in islet grafts, a group of freshly isolated islets and a group of 24 h-cultured islets were included in the study. Cytokine gene expression was higher in islet grafts on day 1 after transplantation than in freshly isolated or in 24 h-cultured islets showing that the transplantation played a role inducing the inflammation at the grafted site. The effect of transplantation procedure was particularly important on IL-1β and TNF-α expression that was much higher on islet grafts than in isolated or cultured islets. In contrast, the impact of transplantation was milder on IL-6 and IL-10 expression that was clearly increased one day after isolation in cultured islets, but only modestly enhanced in islet grafts.

The strong expression of IL-1β on day 1 after transplantation, and its persistently increased expression, even though at lower levels, on days 3 and 7, is particularly relevant due to the well established deleterious effects of IL-1β on beta cells. IL-1β impairs beta cell function, induces beta cell death, and suppresses beta cell replication (12,18,36,39), defects that have all been reported in recently transplanted islets. In addition, IL-1β induction of TNF-α production by beta cells (45) could amplify the inflammatory response in islet grafts. Hyperglycemia, however, did not modify IL-1β gene expression at any studied time after transplantation. Even though glucose induction of IL-1β expression was reported in human islets (20), our results are in agreement with previous data in rodent islets and with the recent report in human islets indicating that glucose does not induce IL-1β production (13, 44). However, hyperglycemia could modify beta cell sensitivity to the deleterious effects of IL-1β (19, 38).

TNF-α gene expression was detected in freshly isolated islets, in agreement with previous data in rodent and human islets (3, 41), and was increased in transplanted islets. TNF-α expression was higher in day 1 hyperglycemic group, but since the difference was not confirmed after adjustment for multiple comparisons, the effect of hyperglycemia on TNF-α expression in islet grafts could not be definitively established. The increased expression of TNF-α in islet grafts had not been previously found (28), and could play a deleterious role on graft survival due to the direct deleterious effects of TNF-α on islet cells (6,17), particularly in combination with IL-1β (19). In addition, TNF-α upregulation of IL-6 production by pancreatic islets (5) could enhance the inflammatory response in islet grafts. However, IL-6 actions depend on cell type, and in rat islets, both IL-6-induced damage (37, 42), and protection (8) has been reported. IL-6 was detected in cultured islets but not in freshly isolated islets, and on day 1 after

transplantation it was increased exclusively in hyperglycemic group. The similar increment in IL-6 expression in 24 h-cultured islets and day 1 normoglycemic grafts compared to the non detectable levels in freshly isolated islets suggests that initial IL-6 expression in islet grafts was mostly related to pre-transplantation processes, but we could not determine whether it was mostly related to the isolation procedure (41), or to factors taking place after islet isolation. A similar consideration is valid for IL-10 expression in islet grafts. On days 3 and 7, IL-6 expression remained as high as in 24 h-cultured islets in hyperglycemic groups, but tended to decrease in normoglycemic groups. Overall, the results indicate that although transplantation did not modify *per se* the expression of IL-6 gene in islet grafts, hyperglycemia enhanced IL-6 gene expression in transplanted islets.

IFN-γ was just barely detected in a few islet grafts, and ambient blood glucose did not modify its expression. In agreement with our results, IFN-γ expression has been previously found in islet allotransplantation, but not or barely in syngeneic grafts (7, 28). However, recent data have implicated IFN-γ in early graft loss in syngeneic islets transplanted to the liver (46), suggesting that the expression of IFN-γ may be dependent on the transplantation site.

Type 2 cytokines IL-4 and IL-10 may have a protective effect on islets exposed to pro-inflammatory cytokines (22,30), and predominant intragraft production of type 2 cytokines has been related to graft acceptance in organ transplantation (2). We did not detect IL-4 gene expression in agreement with previous organ transplantation data showing IL-4 only in allogeneic transplantation (9). In contrast, IL-10 gene expression was significantly increased on day 1 after transplantation both in normoglycemic and hyperglycemic recipients. In normoglycemic groups IL-10 expression decreased subsequently to levels similar to those of 24 h-cultured islets, but it remained increased

in hyperglycemic groups. The data indicates a role for IL-10 against non-specific inflammation in islet grafts, and we suggest that the higher expression in hyperglycemic animals may be in response to a more potent inflammation experienced by transplanted islets exposed to hyperglycemia.

This study has some potential limitations. First, although gene expression analysis provides useful information about the events that take place in islet grafts, post-transcriptional and post-translational modifications may result in discrepancies between mRNA and protein expression. Second, the intensity and characteristics of inflammatory events may be different in other transplantation sites. In particular, inflammation may be higher in islets transplanted into the liver (46). It is remarkable therefore, that we have found a clear induction of inflammatory cytokines using a relatively privileged transplant site and a syngeneic model. These results support the potential important role of early inflammation in islet transplantation. Third, the results found in murine models may not directly apply to human islets. For instance, differences in islet sensitivity to inflammatory cytokines among species have been recognized for long time (10). However, differences in glucose-induced expression of cytokines between murine and human islets (20) have not so far been confirmed (44). Finally, although we have identified that macrophages, a well-known source of pro-inflammatory cytokines, were abundant in islet grafts we have not shown that they produced the cytokines. Moreover, transplanted islet preparations contain other cell types that may contribute to cytokine expression, as suggested by the reported production of IL-1β, IL-6 and TNF-α by beta cells (5,20,45) and acinar cells (31,44), of IL-10 by acinar cells (31), of TNF-α by pancreatic ductal cells (26) or of IL-1 and IL-6 among other by endothelial cells (21).

In summary, we have characterized the non-specific inflammation that takes place in the immediate days after islet transplantation. We have shown that the expression of pro-inflammatory cytokine genes in islet grafts is maximal on day 1 after transplantation, and it is partly modified by the ambient blood glucose. Even though the enhanced expression of cytokine genes in grafts exposed to high blood glucose could be relatively modest, the results suggest that the deleterious effects of hyperglycemia on islet grafts includes the enhancement of non-specific graft inflammation. Understanding the mechanisms of beta cell destruction in islet transplantation will contribute to develop strategies to protect islet grafts.

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Table 1. Characteristics of experimental groups.

Group	Day of graft removal	n	Transplantation		Graft removal	
			Blood glucose (mmol/l)	Body weight (g)	Blood glucose (mmol/l)	Body weight (g)
Normoglycemia	1	6	6.4 ± 0.5	214 ± 9	5.5 ± 0.1	203 ± 8
	3	6	5.7 ± 0.2	235 ± 11	5.5 ± 0.2	233 ± 11
	7	6	6.1 ± 0.2	220 ± 5	5.3 ± 0.1	242 ± 7
Hyperglycemia	1	6	25.8 ± 1.6	195 ± 7	25.9 ± 1.2	195 ± 6
	3	6	30.1 ± 1.3	202 ± 6	22.7 ± 1.9	203 ± 5
	7	6	29.0 ± 2.0	195 ± 6	23.2 ± 2.1	208 ± 3

Values are means ± SEM.

FIGURE LEGEND

Fig. 1. Total RNA obtained from freshly isolated islets (n = 3), 24 h-cultured islets (n = 6), and 500-islet grafts transplanted to normoglycemic (open bars) and hyperglycemic (grey bars) rats and harvested on days 1, 3 and 7 after transplantation. Values are means ± SEM. ANOVA, $p < 0.001$; Fisher's PLSD, * $p < 0.001$ freshly isolated and 24 h-cultured islets vs. all other groups.

Fig. 2. Real-time PCR results for expression levels of target genes on freshly isolated islets, 24 h-cultured islets, and islet grafts on days 1, 3 and 7 after transplantation to normoglycemic (open bars) and hyperglycemic (grey bars) recipients. Gene expression was expressed as ΔC_T. Note that minimum value of target gene expression was 40; and the maximum 0. Values are means ± SEM. N.Q.: Not Quantified due to the very low expression level. Kruskall-Wallis, $p < 0.001$ for each of the 4 cytokines; Mann-Whitney *U* test: * $p = 0.002$ vs. all transplanted groups (IL-1β and TNF-α) or vs. cultured islets (IL-10), # $p = 0.026$ vs. day 1 normoglycemic group (TNF-α) or vs. cultured islets (IL-10), § $p = 0.009$ vs. cultured islets (IL-6 and IL-10), † $p = 0.015$ vs. cultured islets (IL-6).

Fig 3. Macrophage detection in isolated islets and islet grafts. Sections were stained using an anti-CD68 antibody (monocyte/macrophage lineage marker), and visualized with 3,3'-diaminobenzidine tetrahydrochloride on bright field microscope (brown staining). Freshly isolated islets (A) and a typical day 1 graft (B) are shown. Few macrophages were identified in freshly isolated islets (arrows), but abundant macrophages were found in the periphery of islet tissue and in necrotic areas in grafts.

Fig. 1.

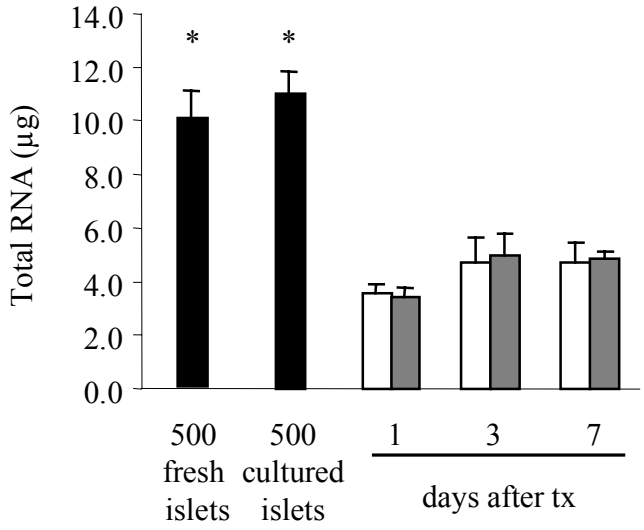

14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Total RNA (µg)
*
*
500 fresh islets
500 cultured islets
1
3
7
days after tx

Fig. 2.

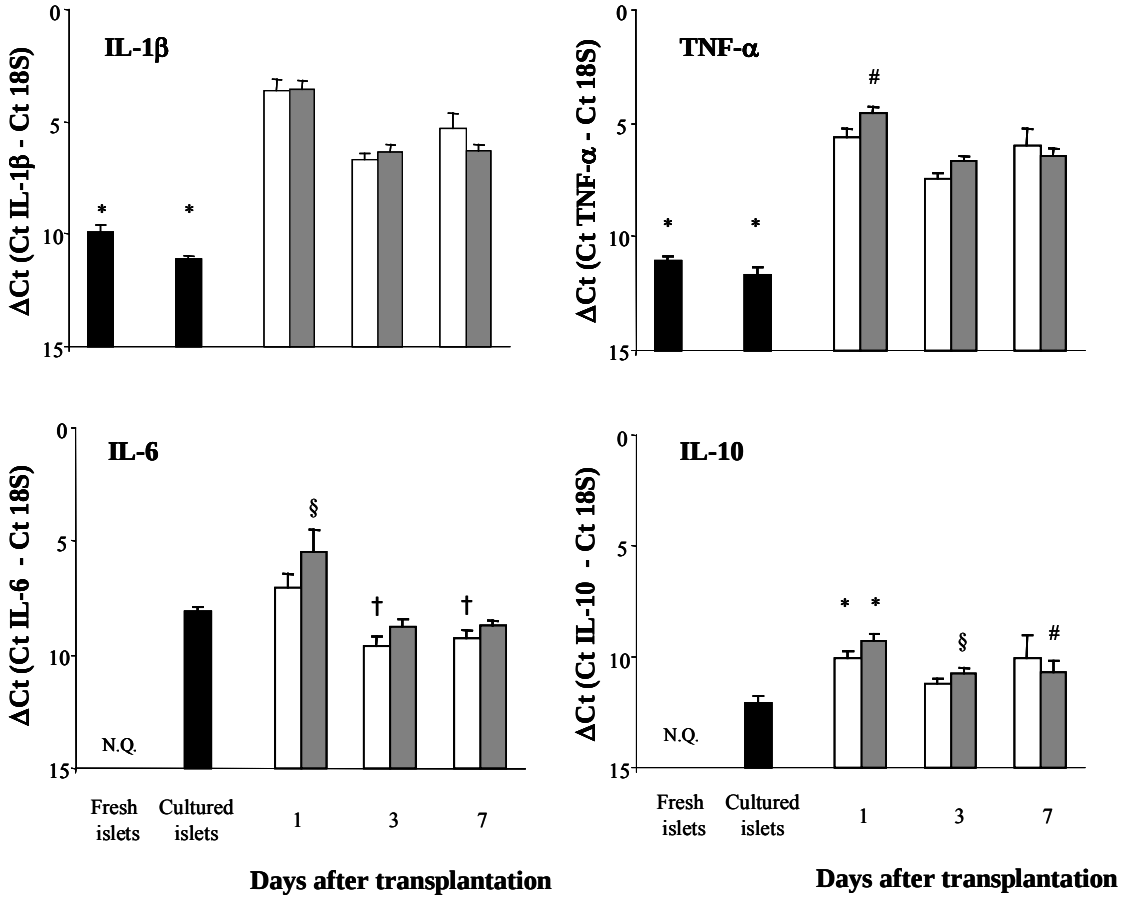
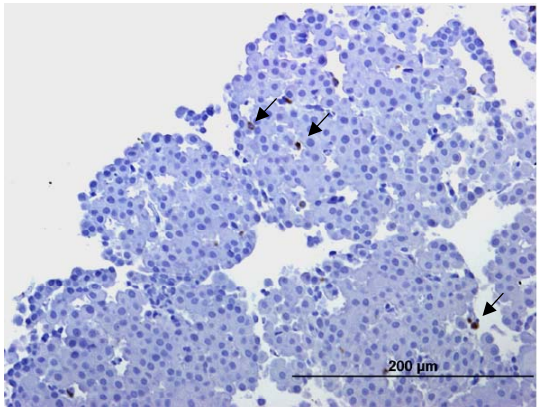

IL-1β
TNF-α
IL-6
IL-10
ΔCt (Ct IL-1β - Ct 18S)
ΔCt (Ct TNF-α - Ct 18S)
ΔCt (Ct IL-6 - Ct 18S)
ΔCt (Ct IL-10 - Ct 18S)
0
5
10
15
N.Q.
Fresh islets
Cultured islets
1
3
7
Days after transplantation

Fig. 3.

A



B