Islet Graft Response to Transplantation Injury Includes Upregulation of Protective as Well as Apoptotic Genes

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Pancreatic islets are particularly vulnerable in the initial days after transplantation when multiple factors converge to damage the islet graft. The aim of this study was to investigate the expression profile of genes involved in damage and protection of β-cells in the initial days after syngeneic islet transplantation. We studied the expression of a set of selected genes involved in apoptosis (Bcl2, BclXs, Bad, BID, and CHOP), cytokine defense, (SOCS-1 and SOCS-3), or free radical protection (Hmox1, Cu/Zn-SOD, Mn-SOD, and Hsp70). Because hyperglycemia has deleterious effects on islet transplantation outcome, we studied its effect on the expression of these genes. Five hundred islets were syngeneically transplanted under the kidney capsule of normoglycemic or streptozotocin-induced diabetic Lewis rats. Gene expression was analyzed by quantitative real-time RT-PCR in grafts 1, 3, and 7 days after transplantation, and in freshly isolated islets. The expression of proapoptotic genes Bid and CHOP, as well as protective genes BclXs, Socs1, Socs3, Hmox1, and MnSod, was maximally increased 1 day after transplantation, and in most cases it remained increased 7 days later, indicating the presence of a protective response against cell damage. In contrast, the expression of Bcl2, Bad, Hsp70, Cu/ZnSod, and Hsp70 genes did not change. Hyperglycemia did not modify the expression of most studied genes. However, MnSod and Ins2 expression was increased and reduced, respectively, on day 7 after transplantation to diabetic recipients, suggesting that hyperglycemia increased oxidative stress and deteriorated β-cell function in transplanted islets.

Key words: Islet transplantation; Apoptosis; Oxidative stress; ER stress; Gene expression; Hyperglycemia

INTRODUCTION

Pancreatic islets are particularly vulnerable in the initial days after transplantation, when more than half of the islet tissue is lost due to increased β-cell apoptosis and necrosis (4,9). This massive cell death increases the islet mass needed to revert diabetes in transplant recipients, and aggravates the shortage of islets available for transplantation (11). The identification of the factors that contribute to transplanted β-cell death/survival in the initial days after transplantation is important in order to develop strategies to improve the preservation of transplanted islets and the outcome of the graft. In this respect, we have previously shown that a brief pretransplantation treatment of islets with the pan-caspase inhibitor z-VAD, which partially reduced transplanted β-cell apoptosis, was sufficient to improve the long-term metabolic outcome of the graft (25), confirming the biological significance of the increased β-cell apoptosis in early islet transplantation.

Several factors converge to damage the islet graft in the initial days after transplantation, among them the process of islet isolation itself, islet hypoxia, nonspecific inflammation of the graft, adverse metabolic condition of the recipient, and the absence of survival factors. However, despite this multifactorial damage a substantial number of β-cells survive and are often capable of restoring normoglycemia in the diabetic recipient, suggesting that transplanted islets may experience the induction of β-cell defense mechanisms able to palliate the damage associated to transplantation and to promote the survival of the graft. We have shown that cytokine expression, and in particular IL-1β, is increased in the graft in the initial days after transplantation (24,26) and that the blockade of IL-1β action has a positive effect on the outcome of the graft, indicating the contribution...
of cytokine-mediated nonspecific inflammation in the initial damage of transplanted islets (35). Although pancreatic β-cells have defense mechanisms against the deleterious effects of cytokines and free radicals, they are considered to be particularly susceptible to cellular stresses due to a low expression of protective genes such as mitochondrial Mn-superoxide dismutase (SOD), cytosolic Cu/ZnSOD (18), or heme oxygenase (Hmox)1 (1). Insulin-producing cells can be protected from oxidative injury by overexpression of stress responsive molecules, such as Hmox1 (19,31,36) or heat shock protein (Hsp) 70 (3). Nevertheless, these defense mechanisms have been rarely investigated in islet grafts.

The aim of the present study was to investigate the expression profile of genes involved in damage and protection of β-cells in the initial days after syngeneic islet transplantation. In particular, we focused our attention on the study of pro- and antiapoptotic genes and on those genes that have been related to defense against, but not exclusively, cytokine-induced β-cell damage, such as members of the newly identified family of suppressors of cytokine signaling (SOCS), antioxidant enzymes, and heat shock proteins. Because we and others have previously found that islets transplanted to normoglycemic, insulin-treated, diabetic recipients have a better outcome than those transplanted to hyperglycemic recipients (4,13,29), we have also compared the expression of these genes in islets transplanted to normoglycemic and hyperglycemic recipients.

MATERIALS AND METHODS

Animals

The Animal Care and Use Committee of the University of Barcelona approved all the in vivo experiments. Male Lewis rats (Harlan, Horst, The Netherlands), 175–200 g body weight, were used as donors and recipients of syngeneic islet transplantation. Animals were housed under conventional conditions in climatized rooms with free access to tap water and standard pelleted food.

Animals were made diabetic by a single IP injection of streptozotocin (STZ) (Sigma Immunochemicals, St. Louis, MO, USA), 65 mg/kg body weight, freshly dissolved in citrate buffer (pH 4.5). After STZ injection, animals were kept hyperglycemic a minimum of 12 days prior to transplantation. Only those rats with blood glucose higher than 20 mmol/L on two consecutive measurements were transplanted. Nonfasting blood glucose was determined between 0900 and 1100 h with a portable glucose meter (Glucocard A, Menarini Diagnostics, Barcelona, Spain).

Experimental Design

On each transplantation day, one normoglycemic non-STZ-injected, and one hyperglycemic STZ-injected, Lewis rat was transplanted with 500 freshly islets from the same isolation. Five hundred islets (absolute number) is an insufficient β-cell mass to restore normoglycemia in STZ-injected rats, and therefore the recipients were expected to remain hyperglycemic after transplantation (2,37).

Grafts exposed to normoglycemic or hyperglycemic conditions were harvested 1, 3, or 7 days after transplantation and processed for RNA purification. The same number of animals was included in each experimental group and specific time point (six groups of animals, four animals in each group). In addition, seven groups of 200 fresh islets, isolated as for transplantation in different days, were also processed for RNA purification.

Islet Isolation, Transplantation, and Graft Harvesting

Islets were isolated by collagenase digestion (Collagenase P, Roche, Mannheim, Germany) of the pancreas as previously described (27). To minimize differences due to endotoxin-induced inflammation during the isolation process (39), all isolations were performed using the same batch of collagenase. Isolated islets were hand-picked under a stereomicroscope two or three times, until a population of pure islets was obtained. Only islets between 75 and 200 µm in diameter were collected, in an effort to reduce hypoxia-mediated β-cell destruction after transplantation (10). Under anesthesia [ketamine (100 mg/kg), diazepam (7.5 mg/kg), and atropine (0.05 mg/kg)] and analgesia (buprenorfine 0.05 mg/kg), the left kidney was exposed through a lumbar incision. A brief capsulotomy was performed and the groups of 500 freshly isolated islets were transplanted under the left kidney capsule. 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 graft, the left kidney was exposed and the graft identified. The kidney capsule surrounding the graft was incised and carefully removed with the graft under the microscope, and the kidney cortex was reviewed after the extraction to confirm that all the graft had been harvested. In the initial days after transplantation the islets do not infiltrate the kidney cortex, allowing the harvesting of the graft with no contamination from kidney cortical cells, as we have previously determined (24). After removal from the kidney, the graft was immediately immersed in lysis buffer for total RNA purification.

RNA Purification and cDNA Synthesis

Total RNA was purified from freshly isolated islets and islet grafts using the RNeasy Mini reagent set with on-column DNase I treatment (Qiagen, Crawley, UK) according to the manufacturer’s protocol. Samples were immersed in lysis buffer and homogenized by mechani-
cal disruption with repeated passing through a Pasteur pipette. The lysate was centrifuged and the nonlysed kidney capsule was discarded. Total RNA was eluted in RNase-free water and tested for genomic DNA contamination. To minimize variation in the retrotranscription reaction, the same amount of RNA (1 μg) with similar quality (260:280 ratio 1.9:2.1) was reverse transcribed simultaneously for all samples. cDNA was synthesized in a final volume of 100 μl using a High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s guidelines. A 10^{-4} dilution was prepared from an aliquot of the resulting cDNA to assess 18S and Ins2 expression, and was stored at −80°C until use.

Relative Quantification of Gene Expression by Real-Time PCR

Real-time PCRs were performed with 1 μl of retrotranscription product (10 ng input or 10 pg for the highly expressed Ins2 mRNA and 18S rRNA) on an ABI Prism 7700 SDS (Applied Biosystems), using Assay-on-Demand FAM-MGB-labeled probes (18S: Hs9999990_1_s1, Bcl2: Rn99999125_m1, BclxL: Rn00580568_g1, Bax: Rn01480160_g1, CHOP: Rn00492098_g1, Socs1: Rn00595838_s1, Socs3: Rn00585674_m1, MnSod: Rn00566942_g1, Cu/Zn Sod: Rn00566938_m1, Hmox1: Rn00561387_m1, Hsp70: Rn00596544_m1, and Ins2: Rn01774648_g1, Applied Biosystems) or Assay-by-Design labeled probes (Bid and Bad, Applied Biosystems). BclxL expression level in fresh islets was very low and 2 μl of cDNA (20 ng input) were used for detection. For relative quantification of gene expression we used the 2^{-ΔΔCt} method (20). 18S was used as endogenous control to normalize the total amounts of cDNA in the samples. Results were then referred to the values of the cDNA from a pool of freshly isolated islets (calibrator) and expressed as the fold increase in mRNA. The adequacy of 18S as endogenous control in our experimental conditions was previously determined using the present set of animals (32). Target and endogenous control genes were amplified in separate wells in the same reaction plate, and each reaction plate included samples from all experimental groups and the calibrator. Data were analyzed using the SDS software (Applied Biosystems).

Statistical Analysis

Results are expressed as mean ± SEM. The effect of transplantation was studied by comparing freshly isolated islets with normoglycemic grafts. The effect of hyperglycemia was studied comparing hyperglycemic grafts with the corresponding normoglycemic grafts at each time point. Normoglycemic and hyperglycemic pairs at each time point were compared with the Mann-Whitney U test, and for comparison of fresh islets and normoglycemic groups the Kruskal-Wallis H test was used (SPSS 11.5.1 program, SPSS, Inc., Chicago, IL). When significant, the Mann-Whitney U test was used to determine specific differences between groups, and subsequently the Hochberg’s Sequential Method was used to adjust the type I error (α) for multiple testing (Multiplicity Program, Department of Biomathematics of the University of Texas M. D. Anderson Cancer Center, Houston, TX). However, the use of adjustments for multiple tests has been seriously questioned (30), and it increases the likelihood of type II errors. Thus, the result of the Mann-Whitney U test is given when it is statistically significant, and it is shown in the figures, and it is followed by the result after the adjustment with the Hochberg’s Sequential Method in the text. The Grubb’s test for detecting outliers was performed using Graph Pad QuickCalcs online calculators for scientists (Graph Pad Software, San Diego California USA, www.graphpad.com). Three outliers were detected among BclxL expression data, corresponding to a fresh islet cDNA expressed 160_g1, MnSod: Rn00566942_g1, Cu/Zn Sod: Rn00566938_m1, Hmox1: Rn00561387_m1, Hsp70: Rn00596544_m1, and Ins2: Rn01774648_g1, Applied Biosystems) or Assay-by-Design labeled probes (Bid and Bad, Applied Biosystems). BclxL expression level in fresh islets was very low and 2 μl of cDNA (20 ng input) were used for detection. For relative quantification of gene expression we used the 2^{-ΔΔCt} method (20). 18S was used as endogenous control to normalize the total amounts of cDNA in the samples. Results were then referred to the values of the cDNA from a pool of freshly isolated islets (calibrator) and expressed as the fold increase in mRNA. The adequacy of 18S as endogenous control in our experimental conditions was previously determined using the present set of animals (32). Target and endogenous control genes were amplified in separate wells in the same reaction plate, and each reaction plate included samples from all experimental groups and the calibrator. Data were analyzed using the SDS software (Applied Biosystems).

RESULTS

Metabolic Evolution

Blood glucose and body weight were similar in all groups when STZ was injected (Table 1). On transplantation day, STZ-injected animals were severely hyperglycemic and had lower body weight that normoglycemic, non-STZ-injected groups. As expected, STZ-diabetic animals remained hyperglycemic after the transplantation of the insufficient β-cell mass, and transplanted non-STZ-injected animals were normoglycemic throughout the study (29).

Gene Expression

The mRNA of all studied genes was detected in freshly isolated islets as well as in islet grafts 1, 3, and 7 days after transplantation. No differences were observed between grafts transplanted to hyperglycemic and to normoglycemic recipients with the exception of MnSod and Ins2 genes (see below).

Apoptosis-Related Genes

The transcript abundance of the antiapoptotic gene Bcl2 and the proapoptotic genes Bax and Bad was unchanged in islet grafts compared to fresh islets (Fig. 1). BclxL expression increased on day 1 (p = 0.011), day 3 (p = 0.011), and day 7 (p = 0.020) after transplantation compared to fresh islets. BclxL expression was also
The expression of the proapoptotic genes Bid and CHOP was increased one day after transplantation ($p = 0.004$, both genes), and the differences remained significant after correction for multiple comparisons ($p = 0.024$). Bid expression remained increased on days 3 and 7 after transplantation ($p = 0.008$, and $p = 0.032$ after multiple comparisons correction), while CHOP expression returned to pretransplantation levels on day 3 post-transplantation.

**Stress Response Genes**

Socs1 and Socs3 expression was increased 1 day after transplantation compared to fresh islets ($p = 0.004$), and to day 3 ($p = 0.014$) and day 7 ($p = 0.014$) islet grafts ($p < 0.05$ in each time point after multiple comparisons correction), and remained increased, although at a lower level, on day 7 after transplantation ($p = 0.008$, and $p < 0.05$ after multiple comparisons correction) (Fig. 2). No statistically significant differences were found in gene expression between normoglycemic and hyperglycemic grafts.

Transcription level of mitochondrial MnSod was increased on day 1 after transplantation compared to fresh islets ($p = 0.004$, and $p = 0.024$ after correction for multiple comparisons) and to day 3 and day 7 islet grafts ($p = 0.014$, and $p = 0.054$ after correction for multiple comparisons) (Fig. 3). MnSod expression on days 3 and 7 after transplantation was similar to that in fresh islets. MnSod expression was increased in grafts retrieved from hyperglycemic recipients on day 7 after transplantation compared with grafts from normoglycemic recipients ($p = 0.021$). In contrast, cytosolic Cu/ZnSod expression did not change throughout the study period, and it was similar in grafts from hyperglycemic and normoglycemic recipients.

The expression of Hmox1 gene (also known as heat shock protein 32) was increased on day 1 after transplantation ($p = 0.004$, and $p = 0.024$ after multiple comparisons correction), and it remained increased on days 3 and 7 ($p = 0.008$, and $p = 0.032$ after multiple comparisons correction) compared to fresh islets (Fig. 3). In contrast, Hsp70 expression did not change significantly after transplantation.

**Insulin**

The expression of the rat insulin gene, Ins2, was reduced in islet grafts compared to fresh islets, but the difference did not reach statistical significance in normoglycemic recipients (Fig. 4). In contrast, Ins2 transcript was progressively reduced in islet grafts transplanted to hyperglycemic recipients, and differences with grafts retrieved from normoglycemic recipients achieved statistical significance on day 7 after transplantation ($p = 0.021$).

**DISCUSSION**

In this study we show that 1 day after transplantation the expression of proapoptotic genes Bid and CHOP, and stress genes Socs1, Socs3, MnSod, and Hmox1 was increased in islet grafts compared to freshly isolated islets, indicating the presence of a significant stress response in the graft in the initial days after transplantation. The expression of several of these genes remained increased, although at a lower level, on day 7 after transplantation. Hyperglycemia did not modify the expression of most of these genes. However, MnSod expression was increased and Ins2 expression was reduced on day 7 after transplantation to diabetic recipients, suggesting that exposure to sustained hyperglycemia increased oxidative stress and deteriorated beta-cell function of transplanted islets.

The Bcl2 family of proteins are universal regulators of apoptosis that control mitochondrial cytochrome c release (7). Translational regulation of Bcl2 family mem-

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**Table 1. Blood Glucose and Body Weight of Experimental Groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>STZ Injection</th>
<th>Transplantation</th>
<th>Day After Transplantation</th>
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<tr>
<td></td>
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<td></td>
<td>1</td>
</tr>
<tr>
<td>Normoglycemic (non-STZ injected)</td>
<td></td>
<td></td>
<td>5.6 ± 0.2</td>
</tr>
<tr>
<td>Blood glucose (mmol/L)</td>
<td></td>
<td></td>
<td>5.3 ± 0.1</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>195 ± 3</td>
<td>251 ± 3</td>
<td>247 ± 3</td>
</tr>
<tr>
<td>Hyperglycemic (STZ injected)</td>
<td></td>
<td></td>
<td>5.9 ± 0.1</td>
</tr>
<tr>
<td>Blood glucose (mmol/L)</td>
<td></td>
<td></td>
<td>27.6 ± 1.7</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>193 ± 3</td>
<td>207 ± 2</td>
<td>206 ± 3</td>
</tr>
</tbody>
</table>

Values are means ± SEM.
Figure 1. Apoptosis-related gene expression profile. qRT-PCR analysis of Bcl2, Bax, Bad, BclxL, Bid, and CHOP mRNAs in freshly isolated islets (n = 7) and islets grafts 1, 3, and 7 days after transplantation to normoglycemic or hyperglycemic recipients (n = 4 in each group). The effect of transplantation was studied comparing freshly isolated islets with normoglycemic grafts. The effect of hyperglycemia was studied comparing hyperglycemic grafts with the corresponding normoglycemic grafts at each time point. Bars indicate mean ± SEM. *p < 0.05 fresh islets versus day 1, 3, and 7 islet grafts (BclxL and Bid expression); #p < 0.05 day 1 islet grafts versus day 3 and day 7 (BclxL and Bid expression), and day 1 versus fresh islets and day 7 grafts (CHOP expression).
Figure 2. Soxs1 and Soxs3 gene expression profile. qRT-PCR analysis in freshly isolated islets (n = 7) and islets grafts 1, 3, and 7 days after transplantation to normoglycemic or hyperglycemic recipients (n = 4 in each group). The effect of transplantation was studied by comparing freshly isolated islets with normoglycemic grafts. The effect of hyperglycemia was studied comparing hyperglycemic grafts with the corresponding normoglycemic grafts at each time point. Bars indicate mean ± SEM. *p < 0.05 fresh islets versus day 1 and 7 islet grafts (Soxs1); **p < 0.05 fresh islets versus day 1, 3, and 7 islet grafts (Soxs3); #p < 0.05 day 1 islet grafts versus day 3 and 7 grafts (Soxs1 and Soxs3).

Suppressors of cytokine signaling (SOCS) are a newly identified family of proteins that suppress the signal transduction process generated by diverse cytokine receptors. SOCS expression is induced by the same cytokine/receptor-mediated signal transduction that they subsequently inhibit, and the system represents an intracellular, fast-acting, negative feedback loop for cytokine signaling. The observed upregulation of Soxs1 and Soxs3 in transplanted islets is consistent with the contribution of cytokines to the acute nonspecific inflammatory response that takes place immediately after transplantation, and is in agreement with recent data showing the increased expression and presence of IL-1β in recently transplanted grafts (24) and the negative impact of IL-1β on the graft outcome (35).

Pancreatic β-cells are particularly susceptible to cellular stresses due to their low expression of antioxidant enzymes such as superoxide dismutase (SOD) (18) or heme oxygenase Hmox1 (1). We have found that the expression of mitochondrial MnSod and Hmox1, but not that of cytosolic Cu/ZnSod, was increased in islet grafts. A direct action of IL-1β on MnSod and Hmox1, but not that of cytosolic Cu/ZnSod, has been previously suggested (5,33). The increased expression of mitochondrial MnSod but not of cytosolic Cu/ZnSod in islet grafts supports the notion that IL-1β plays a significant role in early islet transplantation damage. The expression of Hmox1 was also increased in islet grafts. Upregulation of Hmox1 expression has been found in islet isolation (31) or after an in vitro cytokine challenge (36,41), and
recently an increased Hmox1 gene expression was reported in islet grafts 3 weeks after transplantation (1). Our current results indicate that the expression of Hmox1 is a very initial event in islet transplantation, overall suggesting that Hmox1 may play an important role in the defense mechanisms of the graft. Although a common regulatory pattern for IL-1β-induced MnSod, Hmox1, and Hsp70 expression has been suggested (40), we did not find any significant regulation of Hsp70 expression in our experimental conditions, maybe reflecting differences in the characteristics or intensity of the stimulus contributing to the damage of transplanted islets. Nevertheless, the increased expression of Socs1, Socs3, MnSod, and Hmox1 genes in islet grafts supports the presence of graft inflammation and an early protective response in transplanted islets.

Although hyperglycemia has well-known deleterious effects on the outcome of islet grafts (21–23), we did not find differences in the expression of most studied genes between grafts from normoglycemic and hyperglycemic recipients. We recently observed that hyperglycemia has a minor effect on the expression of several cytokines in islet grafts, and in particular no effect was found on IL-1β expression (26). This is in agreement

Figure 3. Stress-responsive gene expression profile. qRT-PCR analysis of MnSod, Cu/ZnSod, Hmox1, and Hsp70 mRNAs in freshly isolated islets (n = 7) and islets grafts 1, 3, and 7 days after transplantation to normoglycemic or hyperglycemic recipients (n = 4 in each group). The effect of transplantation was studied by comparing freshly isolated islets with normoglycemic grafts. The effect of hyperglycemia was studied comparing hyperglycemic grafts with the corresponding normoglycemic grafts at each time point. Bars indicate mean ± SEM. *p < 0.05 fresh islets versus day 1, 3, and 7 grafts (Hmox1); #p < 0.05 day 1 islet grafts versus fresh islets, and day 1 and 3 grafts (MnSod); Φp < 0.05 day 7 hyperglycemic grafts versus same day normoglycemic grafts (MnSod).
Figure 4. Insulin gene expression profile. qRT-PCR analysis in freshly isolated islets (n = 7) and islets grafts 1, 3, and 7 days after transplantation to normoglycemic or hyperglycemic recipients (n = 4 in each group). The effect of transplantation was studied by comparing freshly isolated islets versus normoglycemic grafts. The effect of hyperglycemia was studied comparing hyperglycemic grafts with the corresponding normoglycemic grafts at each time point. Bars indicate mean ± SEM. *p < 0.05 day 7 hyperglycemic grafts versus same day normoglycemic grafts.

with the lack of differences in Socsl and Socss expression found in grafts from hyperglycemic and normoglycemic recipients. However, our results suggest that sustained hyperglycemia causes oxidative stress and deteriorates β-cell function, as indicated by the increased expression of MnSod and the reduction of Ins2 expression on day 7 after transplantation. It is interesting to notice that the effects on MnSod were not detected until day 7 after transplantation, suggesting that short-term exposure to hyperglycemia could not be deleterious, and in agreement with our previous data indicating that hyperglycemia did not increase β-cell death on day 3 after transplantation (4). In fact, short-term exposure to high glucose has been reported to have a protective effect against β-cell apoptosis (14). Laybutt et al. have reported very recently that chronic hyperglycemia deteriorates β-cell differentiation in transplanted islets (16). They found a nonsignificant reduction in insulin gene expression in islet grafts transplanted to normoglycemic rats (about 60% that of fresh islets), but a profound reduction in transplanted islets exposed to chronic hyperglycemia (16% that of normoglycemic grafts). These results are very similar to ours, and indicate that hyperglycemia has a major deleterious effect on β-cell function of transplanted islets. All together, our results suggest that in the initial days after transplantation, hyperglycemia may not have a major role in early graft inflammation and subsequent islet death, but that has a relevant deleterious effect on β-cell function of transplanted islets.

In summary, the expression of Bclx, Bid, CHOP, Socsl, Socss, Hmox1, and MnSod genes was increased in syngeneic islet grafts initially after transplantation, indicating the presence of a stress response. This transcriptional response provides clues to identify mechanisms of islet cell damage in islet grafts, in particular the contribution of cytokine-mediated nonspecific inflammation, anoikis, oxidative stress, and, maybe, ER stress, that could be used to define strategies to increase the survival of transplanted islets. A more extensive study of differential gene expression can be now undertaken using microarray technologies, and taking advantage of the present results to establish the more appropriate experimental conditions.

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