Interleukin-1 β and inducible form of nitric oxide synthase expression in early syngeneic islet transplantation

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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 expressions of interleukin-1 β (IL-1 β) and inducible form of nitric oxide synthase (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 expressions of *IL-1 β* and *iNOS* genes were determined by RT-PCR. IL-1 β and iNOS mRNAs were detected in islets immediately after isolation, and were upregulated after transplantation. IL-1 β mRNA was ninefold increased on day 1, was still sevenfold increased on day 3 after transplantation, and declined towards pretransplantation levels

on day 7. iNOS mRNA showed a similar pattern of expression to that of IL-1 β : on days 1 and 3 after transplantation it was 14- and 4-fold higher respectively than in freshly isolated islets. In addition, IL-1 β and iNOS were identified in islet grafts and found to be produced mainly by CD68-positive macrophages. A low number of IL-1 β - and iNOS-positive but CD68-negative cells were also identified suggesting that other cell types, in addition to macrophages, were involved in the expression of IL-1 β and NO production in islet grafts. The finding of increased *IL-1 β* and *iNOS* gene expressions 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-1 β plays a role in the extensive β -cell death found in the initial days after islet transplantation.

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Introduction

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 (Biarnés *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 β -cell mass, and to reduce the islet tissue required to achieve normoglycemia in diabetic recipients.

Early islet cell dysfunction and damage take place before immunological rejection of the graft, and although it is more common in xenogeneic and allogeneic transplants, it has also been described in syngeneic islet transplants (Kaufman *et al.* 1990, Biarnés *et al.* 2002), 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 (Vargas *et al.* 1998, Berney *et al.* 2001). An increased expression of inflammatory cytokines may participate in this non-specific inflammation, and induce the functional stunning or destruction of transplanted islets (Ozasa *et al.* 1997, Rabinovitch 1998, Berney *et al.* 2001, Gysemans *et al.* 2003).

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

human islets (Sandler *et al.* 1987, Eizirik 1991, Giannoukakis *et al.* 2000), and to increase β -cell death (Delaney *et al.* 1997, Hoorens *et al.* 2001, Steer *et al.* 2006). *In vitro* observations indicate that the cytotoxic effect of IL-1 β in islet cells involves the induction of nitric oxide synthase (iNOS) and the production of nitric oxide (NO; Hoorens *et al.* 2001, Steer *et al.* 2006). In islet transplantation, it has been suggested that IL-1 β may contribute to early graft dysfunction, but the analysis of *IL-1 β* gene expression has been mostly limited to islets transplanted to non obese diabetic (NOD) mice (Gysmans *et al.* 2000, 2003). Moreover, direct evidence of the presence of the cytokine in islet grafts has 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-1 β* and *iNOS* genes and proteins in the initial days after syngeneic islet transplantation.

Materials and Methods

Experimental design

Animals Male Lewis rats (B&K Universal, UK), aged 7–10 weeks, were used as donors and recipients of transplantation. The recipients were made diabetic by a single i.p. injection of streptozotocin (STZ; Streptozotocin, Sigma Immunochemicals) 65 mg/kg body weight, 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 0900 and 1100 h 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 and fed with tap water and standard pelleted food available *ad libitum*.

Insulin treatment Twelve to fourteen 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 the normal range on transplantation day and on 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 s.c. implant of sustained insulin release (Linplant, Linshin Canada, Inc., Scarborough, ON, 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.

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 μ m 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-PBS, and processed for paraffin embedding.

Gene expression studies

RNA isolation and cDNA synthesis Total RNA was extracted from six groups of 500 freshly isolated islets and from days 1, 3, and 7 islet grafts using RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. Isolated islets and islet grafts were immersed in 350 μ l guanidine isothiocyanate-containing buffer plus 3.5 μ l β -mercaptoethanol. Samples were homogenized 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 diethyl pyrocarbonate-treated water and quantified at 260 nm (DU 640 Spectrophotometer, Beckman, Fullerton, CA, USA).

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

PCR PCR amplification was performed on 2–10 μ l cDNA in a 50 μ l reaction, containing 0.2 μ M of each primer, 200 μ M dNTPs, and 1 U 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 94 °C for 30 s, annealing at different temperatures depending on the gene for 30 s, and elongation at 72 °C for 45 s in a Gene Amp PCR System 9600. PCR cycles were 28 for cyclophilin and 34 for IL-1 β and iNOS. The PCR products

Table 1 Gene specific primers for PCR

	Primer sequence 5'–3'	Annealing temperature (°C)	MgCl ₂ (mM)	Size (bp)
Gene name				
IL-1 β	5' dCCTGTGGCCTTGGGCCTCAA 3' dGGTGCTGATGTACCACTTGGG	60	2	204
iNOS	5' dTCCCCACATTCTTTCCTT 3' dCAAGCGGTCGTTGGGAGTG	58	1·5	204
NKT	5' dATCTTGCCTCCGCTTTCC 3' dAGGTTCCCGACACAGCTG	55	1·4	287
Cyclophilin	5' dAACCCACCGTGTCTTC 3' dTGCCTCTTTCACCTTCCC	55–60	1·4–2	399

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 ³²P-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 specific labeled probe. Post-hybridization washes were performed with 2×SSC/0·1% SDS at 37 °C and membranes were opposed to X-ray film.

Semiquantitative method To compare the relative expression of IL-1 β and iNOS genes between different samples, we used a RT-PCR semiquantitative method. Reaction conditions were standardized for cycle number to observe linear amplification of the PCR products. The PCR for IL-1 β 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 μ l 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 OD of the interest gene relative to the co-amplified 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 novel kidney transporter (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 previously described, and amplified through 40 cycles.

Immunohistochemical studies

Apoptosis detection Three micrometer graft sections were double stained by immunoperoxidase for apoptotic nuclei using the TUNEL technique (*In situ* Cell Death Detection Kit; ApopTag, Intergen, Oxford, UK), and by alkaline phosphatase for the endocrine non- β -cells of the islets, as described (Biarnes *et al.* 2002). β -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. β -Cell apoptosis was expressed as percentage of TUNEL positive β -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.

β -Cell apoptosis was also determined in 9 groups of 100 fresh islets isolated on different days and in 5 pancreata from control Lewis rats. The sections of isolated islets and pancreata were double stained and counted as described for grafts.

Table 2 Oligonucleotide probes for hybridization

	Oligo probe sequence 5'–3'	Hybridization temperature (°C)
Probes		
IL-1 β	5' dATTGTTTGGGATCCACACTCTCCAG	65
iNOS	5' dCGGGATGGCGCCTCTG	58
Cyclophilin	5' dTTTCTCTCCGTAGATGGACTTGCCACCACTGCCA	63

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 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, the sections were blocked with 5% horse serum (Biological Industries, Beit Haemek, Israel), and incubated overnight at 4 °C with mouse anti-rat CD68 antibody (final dilution 1:100; Serotec, Oxford, Oxon, UK). Immunostaining for CD68 was performed using an Immuno Pure ABC Peroxidase Mouse IgG Staining Kit (Pierce, Rockford, IL, 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, the sections were incubated overnight at 4 °C with goat 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, the sections were stained with Sudan Black B (Sigma) for 30 min. The sections were then incubated with donkey fluoroscein isothio-cyanate (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, Glostrup, Denmark). Incubation with DRAQ5 (Biostatus Limited, Leicestershire, UK) was used to dye cell nuclei with a low infrared 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 tumour necrosis factor (TNF)-α + 750 U/ml interferon (IFN)-γ + 10 µg/ml lipo-polysaccharide (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 S.E.M. (mean ± S.E.M.). Differences between means were evaluated by one-way ANOVA. The Fisher's protected least significant difference (PLSD) method was used to determine specific differences between means when determined as significant by ANOVA main effects analysis. A P value of <0.05 was considered significant.

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 five out of six samples from different isolation procedures, and was

Table 3 Characteristics of experimental groups. Values are means ± S.E.M.

Treatment	Day of graft removal	n	First day of insulin treatment		Transplantation day		Day of 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 ^a	–	7	5.7 ± 0.1	167 ± 6	6.0 ± 0.3	200 ± 3	5.7 ± 0.2	233 ± 5

Tx, transplanted; STZ, streptozotocin.
^aIn 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.

Figure 1 Expression of (A) IL-1 β gene and (B) iNOS 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 $\pm$ S.E.M. ANOVA, $P < 0.05$, Fisher PLSD, $*P < 0.01$ between IL-1 β expression in fresh islets and day 1 after transplantation; $^{\dagger}P < 0.05$ among iNOS expression in day 1 after transplantation and all other groups.

upregulated after transplantation. IL-1 β mRNA was ninefold increased on day 1 after transplantation, it was still sevenfold increased on day 3 when compared with fresh islets, and declined towards pretransplantation levels on day 7 (Fig. 1). iNOS mRNA showed a similar pattern of expression as IL-1 β : on days 1 and 3 after transplantation it was 14- and 4-fold higher respectively, than in freshly isolated islets. IL-1 β 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-1 β and iNOS mRNA expressions. In these strictly normoglycemic rats, IL-1 β 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 responsible for the increased IL-1 β and iNOS expressions 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 in 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 (Biarnés *et al.* 2002) were identified.

Figure 2 (A) β -Cell apoptosis in normal pancreas, freshly isolated islets, and day 3 grafts. Values are means $\pm$ S.E.M. ANOVA, $P < 0.005$; Fisher PLSD, $*P < 0.01$ when compared with other groups. (B) Apoptosis in day 3 transplanted β -cell (arrow). Apoptotic nuclei were stained using the TUNEL technique and visualized 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. The 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. Abundant macrophages were found in the periphery of islet tissue and in necrotic areas.

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

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. Thirty-four percent of CD68-positive cells expressed iNOS on day 1 and 8% on day 3. When compared with the rare expression of IL-1 β 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% iNOS-positive cells on days 1 and 3 after transplantation) were found both inside and outside the islet tissue.

Discussion

In this study, we show that the expression of IL-1 β and iNOS genes was already detectable in freshly isolated islets and it increased significantly after transplantation. The expression of IL-1 β and iNOS genes was maximal on day 1 after transplantation and then declined progressively. In addition, IL-1 β - and iNOS-positive cells were found in islet grafts, and macrophages were identified as the main cellular source of both the proteins. The results confirm the presence of an inflammatory process in early islet transplantation, which takes place even in syngeneic transplantation, and suggest that IL-1 β plays a role in the extensive β -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-1 α , IL-2, IL-6, and IFN- γ transcripts in syngeneic and allogeneic islet grafts when compared with freshly isolated islets. However, they could not perform a quantitative comparison of the expression of transcripts on different days, and they did not analyze the presence of the relevant IL-1 β transcript. IL-1 β is a candidate which contributes to early graft inflammation in islet transplantation, but its expression has been investigated almost exclusively in autoimmune models in islet syngeneically transplanted to NOD mice (Gysemans *et al.* 2000, 2003). Cardozo *et al.* (2003) detected the expression of IL-1 β gene in Balb/c islet isografts 8 h after transplantation, but isolated, non-transplanted, islets were not studied and it could not be established whether IL-1 β expression was increased in islet grafts when compared with isolated islets. This is an important question, because previous studies have shown that the endotoxin activity present in collagenase preparations used in islet 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-1 β mRNA in islet grafts on day 1 after transplantation when compared with isolated islets and to days 3 and 7 after transplantation, indicating that the expression of IL-1 β was enhanced by the transplantation process.

This is the first study that directly shows the presence of IL-1 β and iNOS proteins in recently transplanted islet grafts. IL-1 β is the major regulator of iNOS expression in β -cells (Eizirik *et al.* 1992, Karlsen *et al.* 1995), and in rat islets, IL-1 β alone is sufficient to stimulate iNOS expression, and NO production. NO has a fundamental role in cytokine-induced β -cell damage (Eizirik *et al.* 1992, Karlsen *et al.* 1995, Liu *et al.* 2000, Steer *et al.* 2006). Therefore, the expression of iNOS suggests that the presence of IL-1 β in the graft was

Figure 4 Immunofluorescence detection of IL-1 β - and iNOS-positive cells in islet grafts. The sections were double stained for IL-1 β or iNOS, and CD68. IL-1 β expression co-localized mainly with CD68-positive cells (arrows; A), but scarce intra-islet IL-1 β -positive/CD68-negative cells were also found (arrows; B). iNOS expression co-localized mainly with CD68-positive cells (arrows; C), but less abundant iNOS-positive/CD68-negative cells were also found (arrows; D). 'I' indicates islet tissue.

biologically significant. The increased expression of IL-1 β and iNOS genes, along with the identification of IL-1 β 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). Therefore, 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 previously been investigated. We did not detect the kidney-specific gene NKT in graft homogenates, excluding the contamination from kidney tissue thus leaving the graft as the only source of IL-1 β and iNOS mRNAs. Using confocal microscopy, we found that

macrophages were, by far, the main source of IL-1 β and iNOS in islet grafts. The role of macrophages in early graft failure has previously been suggested by the 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-1 β and iNOS expressions in islet grafts, as indicated by 5% of IL-1 β -positive cells and 10–25% of iNOS-positive cells that were CD68-negative. IL-1 β 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 a source of IL-1 β 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, β -cells were the major source of NO in rat and human islets in response to IL-1 β or IL-1 β + IFN- γ respectively (Corbett & McDaniel 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 β -cells to induce β -cell damage (Thomas *et al.* 2002). Nevertheless, the expression of iNOS by the abundant macrophages infiltrating the graft probably contributed also to β -cell damage, similar to that described in other tissues (Bruns *et al.* 2000).

In summary, the increased IL-1 β and iNOS gene and protein expressions in syngeneic islet grafts confirm the presence of a non-specific inflammatory response in the initial days after islet transplantation. Macrophages were the main cellular source of IL-1 β and iNOS, confirming their essential role in early graft inflammation. Considering the well-established cytotoxicity of IL-1 β and NO, they probably contribute to the initial massive loss of transplanted islet cells and the phenomenon of early graft failure.

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