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Immunoprotection of pancreatic islets

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Document Version

Publisher's PDF, also known as Version of record

Publication date:

2004

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Groot, M. D. (2004). Immunoprotection of pancreatic islets: in vitro studies into causes of microencapsulated graft failure. Groningen: s.n.

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CHAPTER SIX

RESPONSE OF ENCAPSULATED RAT PANCREATIC ISLETS TO HYPOXIA

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Cell Transplantation, 12 (8): 867-875, 2003

SUMMARY

Hypoxia contributes to encapsulated pancreatic islet graft failure. To gain insight into the mechanisms that lead to hypoxia-induced graft failure, encapsulated islet function, vitality and cell replication were assessed after two and five days of hypoxic (1% O₂) and normoxic (20% O₂) culture. The mRNA expression levels of Bcl-2, Bax, inducible Nitric Oxide Synthase (iNOS) and Monocyte Chemoattractant Protein 1 (MCP-1) were assessed, as well as the amount of nitrite and MCP-1 in the culture medium. Hypoxia was associated with loss of encapsulated islet function and vitality, but not with an increase in islet cell replication. Loss of vitality was due to necrosis, and only modestly due to apoptosis. Hypoxia was not associated with changes in the Bcl-2/Bax mRNA ratio, but it did increase the expression of iNOS and MCP-1 mRNA. The increased mRNA levels were, however, not associated with elevated concentrations of nitrite nor with elevated levels of MCP-1 protein. The increased iNOS mRNA levels imply a role for NO in the completion of cell death by hypoxia. The increased MCP-1 mRNA levels suggest that encapsulated islets *in vivo* contribute to their own graft failure by attracting cytokine-producing macrophages. The discrepancy between iNOS mRNA and nitrite are explained by the longer half-life of NO during hypoxia. MCP-1 protein levels are underestimated as a consequence of the lower number of vital cells in combination with a higher proteolytic activity due to necrosis. Thus, strategies to eliminate hypoxia may not only improve islet function and vitality, but also reduce the attraction of macrophages by encapsulated islets.

INTRODUCTION

Microencapsulation of pancreatic islets may avoid the need for permanent immunosuppressive drug therapy and open the perspective of xenotransplantation in the treatment of Insulin Dependent Diabetes Mellitus. The commonly applied alginate poly-L-lysine alginate microcapsules provide protection against antibodies and immune cells, while allowing diffusion of insulin and glucose across the capsule membrane. Inherent to the use of immunoprotective capsules is that revascularisation of the islets of Langerhans cannot occur. Absence of blood supply causes attrition and hypoxia, which will lead to metabolic exhaustion and cell death. This explains why that, in spite of successful transplantation experiments in several animal models, encapsulated islet graft survival generally has been limited to periods of several months (12,24,35). Graft failure is a gradual process associated with decreasing islet function, decreasing vitality and an increased proliferation of all islet cells (10).

The aim of the present study was to specifically determine the effect of hypoxia on encapsulated islets. To this end, islet function, islet vitality and islet cell replication rates of encapsulated islets were assessed during normoxic and hypoxic culture. In relation to islet vitality, apoptosis was also studied by measuring the expression of the anti-apoptotic gene Bcl-2 and the pro-apoptotic gene Bax. Bcl-2 is a known inhibitor of hypoxia-induced cell death (31-33). To determine whether hypoxia-induced cell death was mediated through nitric oxide (18,31), the expression of iNOS gene and nitrite formation as a measure of NO production were measured during culture. The effect of hypoxia on the expression of Monocyte Chemoattractant Protein 1 (MCP-1), a factor that may contribute to graft failure by attracting cytokine-producing macrophages, was assessed on both the RNA and protein level.

MATERIALS AND METHODS

Design of the study

Encapsulated islets were cultured for two and five days under hypoxic (1 % O₂, *i.e.* ~7.5 mmHg) or normoxic (20 % O₂, *i.e.* ~150 mmHg) conditions. On day two and day five, islet function, islet vitality and islet cell replication were assessed as well as the mRNA expression of Bcl-2, Bax, iNOS and MCP-1. NO formation was estimated by measuring the amount of nitrite during culture. The amount of secreted MCP-1 protein, lactate and insulin were also assessed in the culture medium.

Animals and islet isolation

Male inbred Albino Oxford rats (Harlan CPB, Zeist, The Netherlands) weighing 297 ± 17 gram (weight \pm sd, $n = 12$) served as islet donors. All animals received care in compliance with the guidelines of the local Animal Care and Use Committee following National Institutes of Health guidelines.

Islets were isolated as previously described (41). Briefly, the donor pancreas was removed after infusion of 10 ml sterile Krebs-Ringer-Hepes solution (KRH, pH 7.4) containing 10 % Bovine Serum Albumin (BSA), through the bile duct. The pancreas was chopped and digested using a two-stage incubation of 20 min at 37°C with successively 1.0, and 0.7 mg/ml collagenase (Boehringer P). Islets were separated from exocrine tissue by centrifugation over a discontinuous Dextran gradient (38) and further purified by handpicking. Islets were cultured overnight in non-treated petri-dishes (Greiner, Alphen a/d/ Rijn, The Netherlands) in portions of 100 islets per 25 cm² in CMRL 1066, containing 10% fetal calf serum (FCS, Gibco), 8.3 mmol/l glucose, 10 mmol/l Hepes and 1% Penicillin/Streptomycin, at 37 °C in humidified air containing 5% CO₂.

Islet encapsulation

Alginate (3.4 % w/v, 60 % mannuronic acid; Keltone LV, Kelco International, London, U.K.) was mixed with cultured islets to a concentration of 1,500 islets/ml. Microencapsulation was performed as described elsewhere (8), except that droplets were incubated in CaCl₂ (100 mmol/l, pH 7.4) for at least five but no more than seven minutes. CaCl₂ was then replaced with KRH and the islet-containing alginate beads (725-775 μ m in diameter) were kept in KRH until all beads were generated. All beads were pooled, washed for one minute with KRH containing 2.5 mmol/l CaCl₂, and PLL coating (poly-L-lysine-HCL, molecular weight 22,000; Sigma) was achieved by incubating for 10 minutes with 0.1 % PLL. Unbound PLL was washed away with Ca²⁺-free KRH (135 mmol/l NaCl) before applying the outer alginate layer by 10 minutes incubation in 10-times diluted alginate solution. Three successive washings with Ca²⁺-free KRH removed the excessive alginate.

Encapsulated islets were cultured in a 48-wells culture plate (tissue culture treated, Costar[®], Cambridge, MA), for 2 and 5 days at 37°C in humidified air with either 5 % CO₂, 20 % O₂ and 75% N₂ (normoxia), or 5% CO₂, 1 % O₂ and 94 % N₂ (hypoxia). Each well contained 90 encapsulated islets suspended in 400 μ l of CMRL 1066 containing 5 % Normal Rat Serum (Gibco Brl), 8.3 mM glucose, 10 mM Hepes and 1 % Penicillin/Streptomycin.

Glucose challenge test

Encapsulated islets were tested for their glucose induced insulin response in three separate samples of 20 islets each. The islets were preincubated for 45-50 minutes in 2 ml Krebs-Ringer-Bicarbonate (KRB, pH 7.4), gassed with 95 % O₂ and 5 % CO₂, containing 0.25 % BSA and 2.75 mM glucose. The *in vitro* insulin secretion was then assessed by three consecutive incubations of 45 minutes in KRB containing (i) 2.75 mM glucose, (ii) 16.5 mM glucose with 0.1 % IsoButylMethylXanthine (IBMX, Sigma), and again (iii) 2.75 mM glucose. At the end of each incubation, the KRB was completely removed and frozen for insulin determination by a radioimmunoassay for rat insulin (Linco, Ede, the Netherlands). The insulin secretory responses of 20 encapsulated islets during 45 minutes were expressed as nanograms of insulin/ml.

Islet vitality staining

Encapsulated islet vitality was assessed by discriminating between apoptotic, necrotic and vital cells. To this end, encapsulated islets were stained with 20 µg/ml Hoechst 33258 (bisbenzimidazole, Sigma) and 10 µg/ml Propidium Iodide (Sigma) in PBS for 10 minutes at 37°C, and scored as described by Saldeen (29). Islet cells with Ho (Hoechst) positive round nuclei were regarded as viable. Round nuclei were regarded as necrotic if both Ho and PI (Propidium Iodide) positive. Highly condensed or fragmented nuclei, Ho positive or both Ho and PI positive, were identified as apoptotic cells. Counting was executed by an independent examiner who scored 700-800 cells at a 400x magnification (Leica DMLB/DC 300F) per experiment. Scores should be regarded as estimations, since superposition of cells makes it difficult sometimes to determine the true cellular status.

Immunohistochemical staining of BrdU

BrdU labeling of the islets cells was performed by incubating the encapsulated islets with 10 µmol/l BrdU (5-Bromo-2-Deoxy Uridine, Boehringer Mannheim) for 24 hours. BrdU-labeled islets were fixed in 2% paraformaldehyde, washed overnight with PBS containing 6% sucrose and dehydrated with acetone for 1 hour prior to Glycolmethacrylate (GMA)-embedding (all incubations at 4°C). Sections of 2 µm were treated with 0.1% trypsin (0.1 M Tris HCl, pH 7.8 + 0.1% CaCl₂) for 15 minutes at room temperature, stained with anti-BrdU (Goat anti BrdU with nuclease, Amersham, Uppsala, Sweden) and finally incubated with a secondary antibody conjugated with peroxidase (Rabbit anti Goat PO, DAKO, Glostrup, Denmark). Peroxidase activity was demonstrated by applying 3-amino-9-ethylcarbazole (AEC) containing hydrogen peroxidase. Hematoxylin was used for staining the cell nuclei. Per condition, approximately 1000 intact

cells were counted. Necrotic and apoptotic cells were excluded from counting, as well as weakly stained cells, since cell repair instead of cell replication could be responsible for staining the cell nuclei. BrdU-positive cells showed a dark red staining of the nucleus. The number of BrdU-positive cells was expressed as the percentage of the total number of BrdU-negative and BrdU-positive cells. BrdU-negative cells were either hematoxylin blue, or colorless as a result of the nuclease activity during the first antibody incubation step.

RT-PCR analysis

Microcapsules were removed from the islets before analysis by shearing the encapsulated islets through a pipette, after a 30 minutes incubation step with 0.5 mM EDTA and 0.25% trypsin at 37°C (7). Batches of 60 decapsulated islets were washed three times with cold PBS and pellet frozen (-80°C). Total RNA was isolated using a Strataprep® Total RNA microprep kit (Stratagene, CA, USA) according to manufacturer's protocol. cDNA synthesis included an initial step in which RNA is incubated with T₁₁ VN oligo's (0.5 µg) for 10 min at 70°C. Subsequently, first strand buffer, dNTP's (final conc. 1 mM), DTT (final conc. 10mM), 200 U M-MLV Reverse Transcriptase (all from Gibco Brl) and 20 U recombinant RNasin® Ribonuclease Inhibitor (Promega) were added and incubated for 50 min at 37°C, after which the reaction was inactivated by a 15 min incubation at 70°C. Since only low amounts of total RNA can be isolated from 60 islets we standardised RT-PCR by using always one-third of the retrieved RNA, in the cDNA synthesis step. Two µl of cDNA was amplified by PCR in a buffer consisting of 0.2 mM dNTP's, 1.5 mM MgCl₂, 1x PCR buffer and 1 U Taq DNA polymerase (all from Gibco Brl). Primer pairs (0.5 µM each) were added after 3 min at 94°C (hot start). PCR was performed on a Mastercycler Gradient apparatus (Eppendorf, Germany) and cycles consisted of 94°C for 40 sec, a primer-pair specific annealing temperature (see below) for 40 sec and 72°C for 40 sec. For each gene, the number of cycles was selected to allow amplification within the linear range. The primer sequences, their annealing temperature, the number of cycles and their respective PCR fragment lengths were; β-actin (253 bp), 24 cycles, 55°C, 5'-AACACCCAGCCATGTACG-3', 5'-ATGTCACGCACGATTTCCC-3'; Bcl-2 (446 bp), 33 cycles, 58°C, 5'-GCTACGAGTGGGATACTGGAGA-3', 5'-AGTCATCCACAGAGCGA TGTT-3' (30); Bax (300 bp), 30 cycles, 52 °C, 5'-AGGATGATTGC TGATGTGGATAC-3', 5'-CACAAAGATGGTCACTGTCTGC-3' (30); iNOS (308 bp), 32 cycles, 57°C, 5'-GACTGCACAGAATGTTCCAG-3', 5'-TGGCCAGATGTTCCTCTATT-3' (5); MCP-1 (306 bp), 28 cycles, 57 °C, 5'-TTCACAGTTGCTGCCTGTAGC-3', 5'-GTGCTGAAGTCCTTAGGGTTGA-3'. Each RNA sample was verified for absence of genomic DNA contamination

by performing RT-PCR reactions in which the addition of the enzyme reverse transcriptase was omitted. Ethidium bromide-stained agarose gels were scanned on Image Master[®] VDS (Pharmacia Biotech, Upsala, Sweden) using LISCAP software. PCR abundance was quantified using Diversity One[™] 1.3 DOS-version (pdi Inc., NY, USA)

Culture sample assessments

Medium culture samples were collected after two and five days of culture and frozen (-20 °C). MCP-1 protein was measured by an Enzyme Immunometric Assay Kit (Titerzyme[®] EIA, Assay Designs Inc.). A polyclonal antibody to rat MCP-1 immobilized on a microtiter plate was used to bind the rat MCP-1 in the sample. The MCP-1 was subsequently incubated with a HO-labeled antibody against MCP-1. Addition of a substrate generates a yellow color that was measured at 450 nm on a microplate fluorescence reader (TL 600, Bio-Tek Instruments Inc.).

Nitrite formation was determined by use of the Griess reaction (13). An equal volume of the Griess reagent was mixed with 50 µl of medium and incubated for 10 minutes at room temperature. The absorbance at 540 was measured on an ultramicroplate reader (EL 808, Bio-Tek Instruments Inc.). Values were calculated from a standard curve ranging from 0.5 to 6.9 µg/ml.

Single samples of media were used for lactate determination by use of a YSI 2300 Stat Plus (Salm & Kipp bv) analyser. Insulin accumulation during culture was determined in 2000 times diluted duplicate medium samples by use of a radioimmunoassay, as mentioned above.

Statistical Analysis

Results were evaluated using a paired Student t-test with a two-tailed distribution (* denotes a significance level < 0.05; # denotes a significance level < 0.10).

RESULTS

Islet function

Encapsulated islet function was assessed by measuring the insulin production of encapsulated islets during culture and by a glucose challenge test after two and five days of culture. On day two, equal amounts of insulin had accumulated in the culture medium of hypoxic (5.4 ± 1.7 ng/ml) and normoxic (5.4 ± 0.4 ng/ml) cultured islets. On day five, different amounts of insulin were found in the culture medium. A significant increase in the amount of insulin was

TABLE 1. The percentage of vital, necrotic and apoptotic cells in encapsulated islets after two and five days of hypoxic (1% O₂) and normoxic (20% O₂) culture (* = P<0.05, two-tailed student t-test, n=6).

	Day 2		Day 5	
	Normoxia	Hypoxia	Normoxia	Hypoxia
Vital	86 ± 5 *	71 ± 8	96 ± 0 *	62 ± 9
Necrosis	12 ± 6 *	23 ± 8	3 ± 0 *	35 ± 10
Apoptosis	2 ± 1 *	6 ± 2	1 ± 0	4 ± 2

found for normoxic cultured islets (towards 13.5 ± 1.1 ng/ml), but not for hypoxic cultured islets (6.4 ± 1.3 ng/ml). Anaerobic metabolic activity was determined by measuring the amount of lactate in the culture medium, which was found to be significantly lower during normoxia (2.8 ± 0.4 mM and 5.3 ± 0.9 mM) compared to hypoxia (4.1 ± 0.4 mM and 7.7 ± 0.4 mM) after two and five days, respectively.

FIGURE 1 shows the basal insulin secretion, the stimulated insulin secretion and the recovered insulin secretion of encapsulated islets during a glucose challenge test after two and five days of culture. Encapsulated islets cultured under normoxic condition responded adequately to both low and high concentrations of glucose. Encapsulated islets cultured under hypoxic conditions did show adequate basal insulin releases, but did not respond to the glucose load (*i.e.* no stimulation).

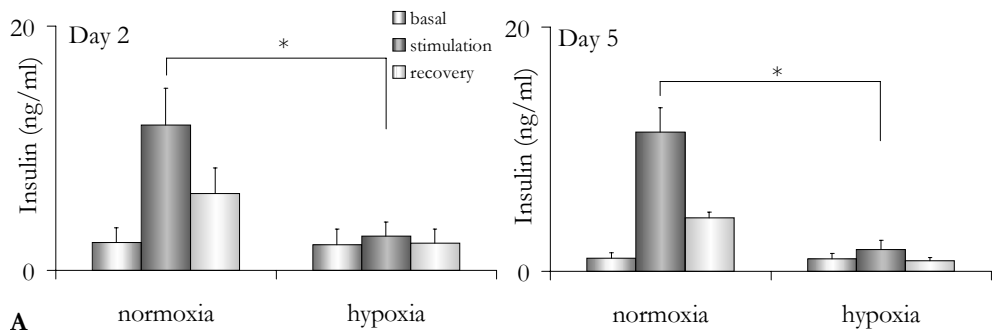


FIGURE 1. The insulin secretion (ng/ml) of encapsulated islets during a glucose challenge test after 2 days (A) and after five days (B) of culture under normal (normoxia, 20% O₂) and low (hypoxia, 1% O₂) oxygen tension (* = P<0.05, two-tailed student t-test, n=6).

Islet vitality

Encapsulated islet vitality was determined by estimating the number of vital, necrotic and apoptotic cells after staining with Hoechst and Propidium Iodide. The vitality score is expressed as the percentage vital cells, which was found to be low after two days of hypoxia ($71 \pm 7\%$) compared to normoxia ($86 \pm 5\%$), and even lower after five days of hypoxia ($62 \pm 9\%$). The loss of vitality was associated with a substantial increase in necrosis ($P < 0.05$ on both days two and five, TABLE 1) and a slight increase in apoptosis ($P < 0.05$ only on day 2).

To gain insight in the molecular mechanism that leads to cell death, mRNA expression levels of the anti-apoptotic Bcl-2, the pro-apoptotic Bax and iNOS, which is associated with both necrosis and apoptosis, were assessed. The expression level of both genes remained similar after two days, but was lower for hypoxic islets compared to normoxic islets for Bcl-2 ($P < 0.05$) and tended to be lower for Bax ($P < 0.10$) on day five (TABLE 2). These decreases did not affect the Bcl-2/Bax ratio, which remained identical for both conditions after two and five days of culture (TABLE 2). The expression of iNOS mRNA had significantly increased after two days of hypoxia, but returned to a level similar to normoxic culture on day five (Figure 2A). The NO production was measured by determining the nitrite formation during culture. Unexpectedly, the amounts of nitrite found in medium of hypoxic cultured islets were similar to the amounts of nitrite found in medium of normoxic cultured islets (FIGURE 2B).

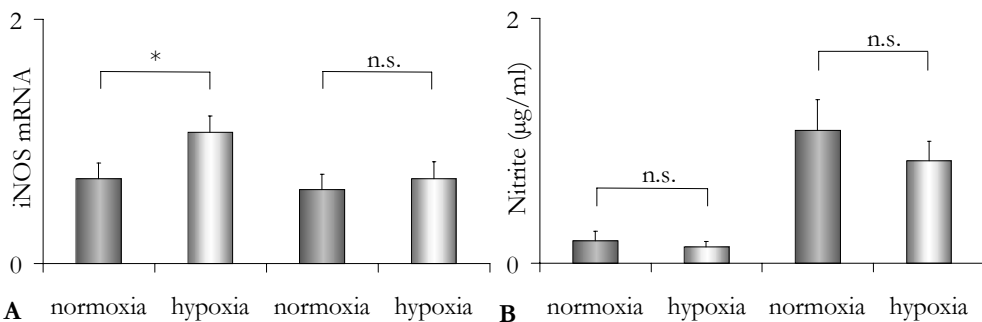


FIGURE 2. The mRNA expression of iNOS relative to β -actin (A), as assessed in encapsulated islets after two and five days of normoxic (20% O_2) and hypoxic (1% O_2) culture (* = $P < 0.05$, two-tailed student *t*-test, $n=6$). The amounts of nitrite (ng/ml) in the culture medium (B), produced by encapsulated islets after two and five days of hypoxic and normoxic culture (n.s. = no significant difference).

TABLE 2. The mRNA expression levels of Bcl-2 and Bax relative to β -actin, and the Bcl-2/Bax ratio. The mRNA expression level of hypoxic cultured encapsulated islets on day 5 was significantly lower compared to normoxia for Bcl-2 (* = $P < 0.05$) and tended to be lower for Bax (# = $P < 0.10$, two tailed student t-test, $n=5$).

	Day 2		Day 5	
	Normoxia	Hypoxia	Normoxia	Hypoxia
Bcl-2	0,5 \pm 0,1	0,4 \pm 0,1	0,4 \pm 0,1	* 0,3 \pm 0,1
Bax	1,0 \pm 0,2	0,8 \pm 0,2	1,1 \pm 0,3	# 0,6 \pm 0,1
Bcl-2/Bax	0,5 \pm 0,1	0,6 \pm 0,1	0,5 \pm 0,1	0,5 \pm 0,2

Islet cell replication

To test whether hypoxia affects the proliferation of islet cells, the islet cell replication during 24 hrs was assessed. The replication rate is the number of BrdU-positive cells expressed as the percentage of the total number of BrdU-negative and BrdU-positive cells. Since necrotic and apoptotic cells were excluded from counting, these values represent the percentage of replicating cells of the vital part of the islets. No significant differences in the encapsulated islet cell replication rates were found after two days (5.8 % \pm 1.9 and 4.3 % \pm 1.7 per 24 hrs) and after five days (2.1 % \pm 0.4 and 3.0 % \pm 0.9 per 24 hrs) of normoxic and hypoxic culture respectively.

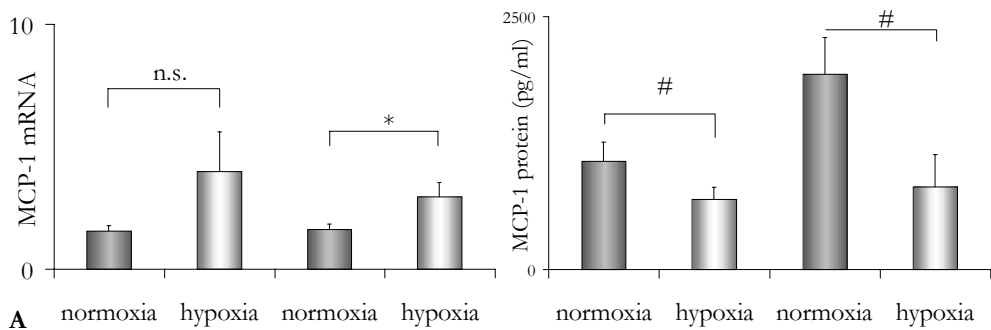


FIGURE 3. The mRNA expression of MCP-1 in encapsulated islets (A) and the amount of MCP-1 protein (pg/ml) in the culture medium (B) after two and five days of hypoxic (1% O₂) and normoxic (20% O₂) culture (* = $P < 0.05$; # = $P < 0.10$, two-tailed student t-test, $n=5$).

MCP-1 in response to hypoxia

To determine the relation between hypoxic stress and islet factors that may induce peri-capsular overgrowth by macrophages *in vivo*, the MCP-1 production of encapsulated islets was measured. MCP-1 mRNA expression was determined by RT-PCR and MCP-1 protein levels in the culture medium were assessed by EIA. RT-PCR results show that the expression of MCP-1 mRNA is elevated under hypoxic stress after two days (2.6 fold, not significant) and after five days (1.8 fold, $P < 0.05$) of culture (FIGURE 3A). MCP-1 protein levels (FIGURE 3B), however, were similar for encapsulated islets under normoxic and hypoxic culture, with a tendency to be lower for hypoxic islets rather than higher ($P < 0.10$ on both days).

DISCUSSION

Hypoxia is regarded as one of the main causes of encapsulated islet graft failure. The present study is an analysis of encapsulated islets during hypoxic stress *in vitro*, in which we show that hypoxia leads to dysfunction and loss of vitality, but not to increased proliferation of the islet cells.

Encapsulated islet function, as expressed in insulin secreting capacity, was severely affected by hypoxia. Glucose challenge of hypoxic cultured islets did not result in elevated insulin secretion levels, and the basal insulin secretion, too, remained not unaffected. Although the basal levels were identical for hypoxic and normoxic encapsulated islets, the amount of insulin on day five was lower in the hypoxic than in the normoxic culture medium. These findings are consistent with those of Dionne *et al.* who found the second-phase insulin secretion rate during perfusion to be almost completely eliminated at a pO_2 of 10 mmHg (= 1.3% kPa or 1.3% O_2), while the basal secretion rate was only slightly reduced (11). The maintenance of basal insulin secretion capacity can be explained by the lower oxygen consumption of islets during the basal insulin secretion phase (3,26,40). In the present study, the stimulated insulin secretion level was reduced to a basal level already after two days, while it decreased gradually over a period of 2-3 months in a previous *in vivo* study from our laboratory (10). The differences between these *in vivo* and the present *in vitro* results may be explained by the difference in the degree of hypoxia. The pO_2 in the peritoneal transplantation site was found to be in the order of 26-77 mmHg (= 3.5 – 10% O_2) (16,17,19,27), which is higher than the level of hypoxia applied here (1% O_2 = 7.5 mmHg). We chose severe hypoxia (1% O_2) since the pO_2 in the peritoneum is low, and may locally even approximate zero according to Zimmerman *et al.* (44). It is reasonable to assume that the oxygen tension in the encapsulated islets is even lower than the 1% O_2 in the overlying gas phase. As a consequence, the core of

the islets is likely to be anoxic rather than hypoxic, which gives an explanation to the high loss of vitality and the high loss of function.

The reduced vitality was mainly associated with necrosis and to a lesser extent to apoptosis. The cell sensitivity for apoptosis did not change via Bcl-2 or Bax, since their ratio was not affected by hypoxia. The individual expression levels, however, were reduced for both Bcl-2 and Bax to half of the initial value after five days of hypoxia. Since Bcl-2 family members are more expressed in the centrally located beta cells than in the peripherally located alpha cells (14), the observed reduction of Bcl-2 and Bax expression may well reflect the decrease in the number of beta cells as a result of central necrosis. Hypoxia is reported to induce both necrosis and apoptosis, and the proportion of these two modes depends on the cell type, the duration, and the severity of the hypoxic stress (31). (4) Moritz *et al.* recently reported complete disintegration of the islet core after 48 hours of hypoxia (1% O₂), and they found hypoxia to be particularly related to apoptosis after 6 hours (22). It is thus possible that in the present study also apoptosis was the predominant mode of cell death during the first hours of hypoxia. Since apoptotic cells can become necrotic if they are not engulfed by phagocytes, it remains unclear to what extent the high percentage of necrosis after 48 hours was directly induced by hypoxia, or a secondary product of hypoxia-induced apoptosis.

The massive cell death during hypoxia, which consisted mainly of necrosis, was associated with an increase of iNOS mRNA on day two, which implies a role for NO in the completion of cell death. The observed discrepancy between the high iNOS mRNA and the low nitrite levels as a measure for NO production *in vitro* has been described before (1,2,39). Low oxygen tension increases the half-life of NO, which enhances the chance of NO to react with scavengers, or to escape from the medium by partitioning in the overlying gas phase (1,2). This means that despite the low levels of nitrite measured, high levels of NO may have been present in the medium.

Increased islet cell proliferation after transplantation has been repeatedly reported and is regarded as a functional compensation for an insufficient islet mass (10,21,23,36). Increased proliferation may also be the result of signals released by necrotic cells (20). However, although hypoxic cultured islets became necrotic, hypoxic and normoxic cultured islets showed similar replication rates in the present study. The proliferation rates of both hypoxic and normoxic cultured islets in the present study (2-6%) are in the same range as the proliferation rates observed after transplantation in a previous *in vivo* study (4-6%), which were increased as compared to the proliferation rate in the native pancreas (10). High replication rates *in vitro* have been observed before and may well be explained by the amount of glucose in the culture medium. Sjöholm *et al* showed that rat

islets had a replication rate of 3.0% if cultured with 3.3 mM glucose and 5.8% if cultured with 11.1 mM glucose (34). The initial glucose concentration was 8.3 mM in the present and in a previous study from our laboratory, where free and encapsulated islet cell replication rates were found to be 6.1% and 6.5%, respectively, after two days of culture (10). This is also in line with the results of Hayek *et al.* who showed that human islet cell replication was 4.7% after three days of culture at 5.5 mM glucose (15).

Hypoxia was associated with an increase in the mRNA expression level of MCP-1, but this increase was not detected on a protein level. On the contrary, the amount of MCP-1 protein tended to be lower in the culture medium of hypoxic as compared to normoxic islets. This apparent contradiction can be readily explained, since the number of vital cells able to produce MCP-1 is considerably lower during hypoxic culture. If we correct the amount of secreted MCP-1 in line with the vitality scores, it appears that at least equal amounts of MCP-1 were produced under hypoxic and normoxic conditions. The MCP-1 production is probably even higher with hypoxia since a higher proteolytic activity due to necrosis may well be responsible for an underestimation of the actual amount of MCP-1 protein. A high MCP-1 mRNA expression can be understood as a signal of islets to promote angiogenesis in order to solve the lack of oxygen (28). In a transplantation setting, however, the chemotactic activity of MCP-1 may contribute to graft failure by attracting cytokine-producing macrophages (25).

Thus, hypoxia is a crucial factor in encapsulated islet graft failure. It leads to loss of islet function and vitality, but not to pronounced proliferation of islet cells. Obviously, strategies to reduce the hypoxic stress on islets are required for successful transplantation. One approach is to improve the blood supply by the use of a prevascularised implantation site (9). It may not only improve graft function by eliminating hypoxia, but it may also diminish the chemotactic process of attracting peritoneal macrophages that contribute to graft failure through pericapsular overgrowth and the production of cytokines. Another approach would be to increase the islet resistance against hypoxia, either by using hypoxia resistant piscine islets from Tilapia, a tropical teleost fish (42,43) or by e.g. ischemic preconditioning (6,37). Conceptually, these approaches may very well be combined.

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