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Islet morphology and function following foetal rat pancreatic transplantation

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Summary

The restoration of endocrine and metabolic function remains the ultimate goal in the treatment of diabetes mellitus. Foetal pancreatic transplantation, because of the growth potential of especially the endocrine component, is an attractive method for endocrine replacement. Intravenous injection of 50 mg/kg streptozotocin into the tail vein of WAG rats resulted in the rapid onset of uncontrolled diabetes in 90% of recipients, requiring insulin support for survival. Transplantation of isogeneic (WAG ➪ WAG) foetal pancreata successfully reversed hyperglycaemia in 10 out of a group of 12 diabetic animals, lowering whole blood glucose levels from 13.47 ± 1.54 mmol/l (with insulin support) to 7.63 ± 0.276 mmol/l (without insulin) within the first week post-transplantation. Other clinical features of diabetes were also reversed. At one and nine months post-transplantation, non-fasting whole blood glucose levels had normalised to 6.37 and 6.59 mmol/l (normal = 6.42 mmol/l). Intravenous glucose tolerance tests of the transplanted groups showed a return to basal values after 30 and 40 minutes respectively. Insulin secretion in response to glucose load yielded peak insulin values of 23.93 µIU/l and 30.81 µIU/l (normal = 70.93 µIU/l), at one and nine months post-transplantation, compared to 3.18 µIU/l of the diabetic controls.

Histology of the grafts showed the presence of well-developed islets of Langerhans while the exocrine component had atrophied. Immunocytochemistry showed that the islets consisted mostly of insulin-positive β-cells. Islet β-cell volume of the grafts was not significantly different to the normal controls but morphometry showed a significant reduction in graft islet α-cell volume and α-cell size compared with normal pancreas islets of Langerhans. The results confirmed that although aberrations in the development of transplanted islets were noted, recipients of rat foetal pancreas transplants in this laboratory diabetic model were able to effectively reverse the clinical signs of diabetes in the short- and longer-term.

Key Words: Endocrine – Diabetes – Pancreatic – Transplantation – Islet – Foetal

Introduction

Restoration of endocrine function remains the ultimate goal in the management and treatment of type 1, insulin-dependent diabetes mellitus. Pancreatic transplantation has met with increased success and the need for such replacement, especially in diabetics with end-stage renal disease, has grown rapidly, outstripping the supply of donor organs (Cicalese et al., 1999; Sutherland et al., 1999; Gruessner et al., 1998; Bouwens et al., 1998). A successful pancreas graft, provided rejection is prevented, restores normoglycaemia and abrogates some of the serious metabolic complications common to diabetes (Yderstraede et al., 1995, 1998; Weitgasser et al., 1995; Tajra et al., 1998; Brown et al., 1984; Mullen et al., 1976; McEnvoy and Hegre, 1978,

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1979; Feldman et al., 1980; Mandel et al 1982; Du Toit et al., 1998; Kemp et al., 1973). New immunosuppressive drugs and treatment protocols have alleviated the diabetogenic side-effects seen with conventional macrolide and steroid-based immunosuppression (Gummert et al., 1999; Gruessner et al., 1998). Pancreatic transplantation, for treating type 1 diabetics with complications, has benefited from new immunosuppression protocols (Gummert et al., 1999). Islet transplantation in humans is problematic and has not met with the same success as whole pancreas transplantation (Hiller et al., 1991). Isolation of sufficient numbers of viable islets and other technical aspects have been the stumbling block in clinical transplantation. Foetal pancreas transplantation as a source of endocrine replacement offers several attractive benefits which include ease and safety of transplantation and the potential of the foetal pancreas endocrine component to grow and differentiate (Brown et al., 1984). The preferential early development of the endocrine component in the foetal pancreas counteracts the need for isolation of the islets from exocrine tissue shown to be necessary for successful nonvascularized islet engraftment (Brown et al., 1984; McEnvoy and Hegre, 1978, 1979).

Isolated pancreatic islets transplanted under the kidney capsule are also capable of sustaining endocrine function in the long-term isograft models (Hiller et al., 1991; Yderstraede et al., 1995; Weitgasser et al., 1995; Feldman et al., 1980). In this study we evaluated the short- and long-term endocrine function and islet morphology following isogeneic renal sub-capsular foetal rat pancreatic transplantation.

Materials and Methods

Experimental procedure

Inbred 5 month old, adult pathogen-free (SPF) male WAG rats (a sub-strain of Wistar rat) were obtained from the animal facility, University of Stellenbosch, Medical School. All procedures were performed according to an ethically approved experimental protocol, protocol no. 87/072, registered at the University of Stellenbosch. Normal day-night schedules where maintained within a stress-free environment. Rats, randomised into experimental groups (Table 1), had free access to water and food at all times. All surgical procedures and catheterization were performed under general anaesthetic using 0.1 ml of a 10 mg/ml ketamine hydrochloride (Ketolar®, Parke Davis, Sandy, USA) intramuscular injection and diethyl ether inhalation. To negate the anaesthetic effect on glucose and insulin values, specimen collection was always performed under general diethyl ether and ketamine hydrochloride anaesthesia (Aynsley-Green et al., 1973; Moin et al., 1995)

Diabetic model

A single intravenous injection of 50 mg/kg streptozotocin (Zanozar®, Pharmacia & Upjohn, Kalamazoo, Michigan, USA) was injected via a catheter (Abocath, Abbot Laboratories, USA) into the tail vein of adult (320–370 g) WAG rats. Non-fasting whole blood glucose (WBG) monitoring (Glucometer, Ames, Isando, South Africa) was performed daily and parenteral insulin (Actraphane, Novo, Johannesburg, South Africa) given according to the schedule (Table 2) in order to avoid uncontrollable ketoacidosis.

Foetal rat pancreas transplantation

Because exocrine tissue development in the rat predominantly begins after 17 days of gestation, foetal pancreata of 16–18 days gestation were used for our transplantation studies (Brown et al., 1984; Hegre et al., 1973).

WAG foetal pancreata of between 16 and 18 days of gestation were obtained from anaesthetized (ketamine and ether) time-bred pregnant donor WAG female rats by laparotomy and caesarian section. The foetal pancreata were carefully harvested from each donor using an operating microscope (Carl Zeiss, Germany). Harvested foetal pancreata were kept on ice in RPMI medium (Gibco, Scotland). Cold ischaemic times were kept below 45 minutes.

A 2–3 cm midline laparotomy incision was performed in the recipient and the kidney lifted into view. After exposing the kidney from surrounding organs a small incision, just deep enough to penetrate the capsule, was made and a subcapsular pocket prepared by gently separating the kidney capsule from the underlying cortex with a fine curved forceps. Bleeding was controlled by applying gentle pressure where necessary. The pocket was then rinsed with sterile physiological saline. Between 4-8 foetal pancreata were positioned in the subcapsular pocket. The kidney capsule was left unsutured and

Table 1.- Experimental groups.

Groups	Treatment	n =
(1) Normal control	no surgical intervention	7
(2) Diabetic control	STZ 50 mg/kg and insulin support, no endocrine replacement	6
(3) 1 month post-Tx	STZ 50 mg/kg and FRPT*	6
(4) 9 months post-Tx	STZ 50 mg/kg and FRPT*	4

STZ: Streptozotocin, * FRPT: Isogeneic foetal rat pancreatic transplantation.

the kidney allowed to return to its position. Before closing the abdomen, 3 ml of warm (±30°C) sterile Ringers lactate (SABAX, Port Elizabeth, South Africa) was placed in the peritonial cavity and the laparotomy incision sutured using 4/0 Dexon II® (Davis and Geck, Isando, South Africa). The animals were then returned to clean cages and kept warm.

Metabolic studies

Metabolic cages were used to assess 24 hour water intake and urine output. Multistix® 10 SG urine test strips (Bayer Corporation, USA) were used to screen the urine samples for glucose, ketones and proteins.

Monitoring of whole blood glucose (WBG)

Daily monitoring of WBG and insulin support in transplanted recipients continued until the animals were able to maintain random WBG levels of < 10 mmol/l. Once normoglycaemia was restored, glucose testing was done twice weekly for three weeks and weekly thereafter. Normoglycaemia was defined as a WBG of < 8.0 mmol/l for three consecutive days without any insulin.

Intravenous glucose tolerance test (IVGTT)

After collecting a basal WBG sample, a single bolus of 50% dextrose solution at a dose of 0.5 mg/kg was injected intravenously as a steady bolus infusion over 20 seconds. WBG determinations were done from the 1, 2, 3, 5, 10, 15, 20, 30, 40, 50 and 60 minute specimens. The whole IVGTT procedure was performed under general ketamine hydrochloride and di-ethyl ether anaesthesia.

Insulin response to IVGTT

Serum insulin determination was performed on aliquoted specimens. Due to the short collecting intervals, at the beginning of the test, the amount of blood collected ranged from 100–200 µl per specimen. Since the Coat-a-Count® ^{125}I insulin radioimmunoassay method requires 200 µl per sample, 100 µl of processed insulin-free human serum (zero standard supplied with the kit) was added to 100 µl of the specimen and the insulin concentration calculated according to the dilution factor. To increase sensitivity at the low end of the curve an additional 1/2 x 1 standard was added. The Coat-a-Count® tubes were then counted in a Beckman® gamma counter for one minute and the insulin concentrations determined on a spline curve.

Histological evaluation

Established grafts were harvested for histology and immunocytochemical evaluation at one and nine months post-transplantation. Control adult and foetal tissue were included. Serial sections of the intact subcapsular pancreas grafts and kidney interface were produced and stained with haematoxylin and eosin. The presence of insulin, glucagon, somatostatin, pancreatic polypeptide (PP), S100 protein, neuron-specific enolase and neurofilament protein was determined immunocytochemically on paraffin sections.

Immunocytochemical method

Serial formaldehyde-fixed paraffin sections were dewaxed in xylene, hydrated in ethanol and washed in distilled water. Endogenous peroxidase was blocked by 1.5% hydrogen peroxide in methanol for 10 minutes and the sections washed in phosphate buffered saline (PBS) at pH 7.6. Sections were incubated with normal serum for 20 minutes in order to block non-specific binding sites. Primary antibodies used as listed in Table 3 were then applied to the sections for 60 minutes.

Method controls in which the primary antibody was replaced with normal mouse serum (DAKO cat no: X0910, Glosstrup, Denmark) were included. Sections were then washed in three changes of PBS and the buffer drained. Biotinylated link-antibody (DAKO, LSAB® 2 kit cat.no. K607, Glostrup, Denmark) was applied for 20 minutes followed by further washing with PBS as previously described. Sections were then incubated for 20 minutes with the peroxidase conjugated streptavidin-biotin complex (DAKO, LSAB® 2 kit cat.no. K607, Glostrup, Denmark) and washed as previously described. Immunoreactivity was demonstrated by incubation for 10 minutes with 0.05% 3,3'-diaminobenzidine tetrachloride (DAB) in PBS, pH 7.6 to which 160 µl of 3% H_2O_2 was added per 50 ml of the DAB solution. Sections were counterstained with Mayers haematoxylin, dehydrated, cleared and mounted in DPX.

Statistical analysis

All the results were expressed as mean ± SEM. Statistical analysis of the results were done by using the Mann Whitney-U test or Wilcoxon signed rank test where appropriate. A p-value of < 0.05 was deemed to be statistically significant. Correlation between groups was assessed by the Spearman's rank correlation coefficient test.

Morphometric analysis

The Image analysis system consisted of a Zeiss MC63 (Germany) light microscope with Pulnix CCD attached to a pentium computer running HLImage ++97 image analysis software. The image analysis software was calibrated for the objective used so that all measurements were expressed in µm or μm^2.

Each section was analysed using a x10 objective lens. We used adjacent fields, analysing the

area of tissue falling within the eyepiece graticule square. Nuclei of immuno-positive staining β- or α-cells were counted in order to obtain absolute cell numbers. Cell population area measurements were obtained by colour thresholding. Islet area was determined by tracing the around the islet with the computer mouse. Results were exported to a spreadsheet (Excel 97, Microsoft) for data analysis.

Results

Diabetic rat foetal pancreas transplant model

A single intravenous dose of 50 mg/kg streptozotocin, injected into the tail vein of an adult WAG rat induced rapid hyperglycaemia in 90% of the recipients, i.e. non-fasting whole blood glucose > 10 mmol/l, within 2 days after the injection. Daily subcutaneous insulin injections (Table 2) of between 1–5 U/day were required to ensure survival and maintain a non-fasting whole blood glucose level of below 20.0 mmol/l. One week after inducing diabetes, non-transplanted diabetic control rats had blood glucose values of 13.47 ± 1.54 mmol/l while on insulin support.

Table 2.- Insulin treatment schedule pre-transplantation and for diabatic controls.

Non-fasting WBG	Insulin
< 10 mmol/l	No insulin
10 –15 mmol/l	1 Unit
15.1 – 20 mmol/l	2 Units
>20,1 mmol/l	3 Units

Table 3.- Inmunocytochemical Primary Antibodies.

Antigen	Dilution	Source
Insulin (p)	1 : 80	DAKO cat no: A 0564, Glosstrup, Denmark
Glucagon (p)	1 : 50	Novocastra Lab cat no: NCL-GLUCp, Newcastle, UK
Somatostatin (p)	1 : 200	DAKO cat no: A0566, Glosstrup, Denmark
PP (p)	1: 200	DAKO cat no: A 0619, Glosstrup, Denmark
neurofilament protein (m)	1 : 250	DAKO cat no: M0762, Glosstrup, Denmark
neuron-specific enolase (m)	1 : 250	DAKO cat no: M0873, Glosstrup, Denmark
S100 (p)	1 : 100	DAKO cat no: Z0311, Glosstrup, Denmark

(p) = polyclonal; (m) = monoclonal). All antibodies known to X-react with specific rat peptides.

Foetal pancreas transplants were effected within 3-11 days after Streptozotocin injection. Between 4 and 8 isogeneic foetal pancreata of between 16 and 18 days gestation were transplanted under the capsules of both kidneys. Where technical problems were encountered i.e. excessive subcapsular bleeding or tearing of the capsule, only one kidney was used as the recipient organ. Whole blood glucose monitoring and subcutaneous insulin support was maintained until the grafts were able to reverse the diabetic state: i.e. maintain a non-fasting whole blood glucose level of < 10.0 mmol/l for three consecutive days without insulin support. This occurred 4-15 days post-transplantation (average 8 days) after which no further insulin support was necessary. The first week following the withdrawal of insulin support the transplanted rats (groups 3, 4) had mean non-fasting whole blood glucose levels of 7.63 ± 0.276 mmol/l, with a range of 4.1-12.4 mmol/l. These values were still significantly higher than the normal non-fasting control mean values of 6.42 ± 0.055 mmol/l (Grp 1; p = 0.02).

Of the 12 diabetic animals that received FRPT in groups 3 and 4, two failed to reverse their hyperglycaemia and were lost at 3 and 21 days respectively. One animal, after reversing the hyperglycaemia at 6 days post-transplantation had a brief relapse on days 13 and 14 post-transplantation with WBG values of 17.4 mmol/l and 16.7 mmol/l, requiring insulin (2 U/day); thereafter the animal remained normoglycaemic.

Experimental animal weights (Table 4)

Mean weights of the control animals were 343.7 g ± 2.96 at 5 months and 454.5 g ± 9.84. at 15 months. Animals of the diabetic control group showed signs of general wasting and dehydration, with a 12% mean bodyweight loss at 1 month.

At 1 month post-transplantation, the animals still showed a mean bodyweight loss of 11%. By 9 months post-transplantation the animals appeared to mature and develop normally, increasing their mean weight to 417.2 g ± 35.9, which was still significantly below the normal controls at 454.5 g ± 31.1 (Grp 4 vs Grp 1; p = 0.06).

Metabolic profile of experimental groups (Table 5)

Metabolic profiles of the control animals included a mean water intake of 21.3 ± 6.8 ml/24hrs and a urine output of 7.9 ± 1.3 ml/24hrs. The profiles of group 2, the diabetic control group at 1 month, included a mean water intake 55.8 ± 10.9 ml/24 hrs (Grp 1; p = 0.002) and a urine output 29 ± 6.4 9 ml/24 ± Grp 1; p = 0.008) with glucose 4+ and traces of protein present. By one

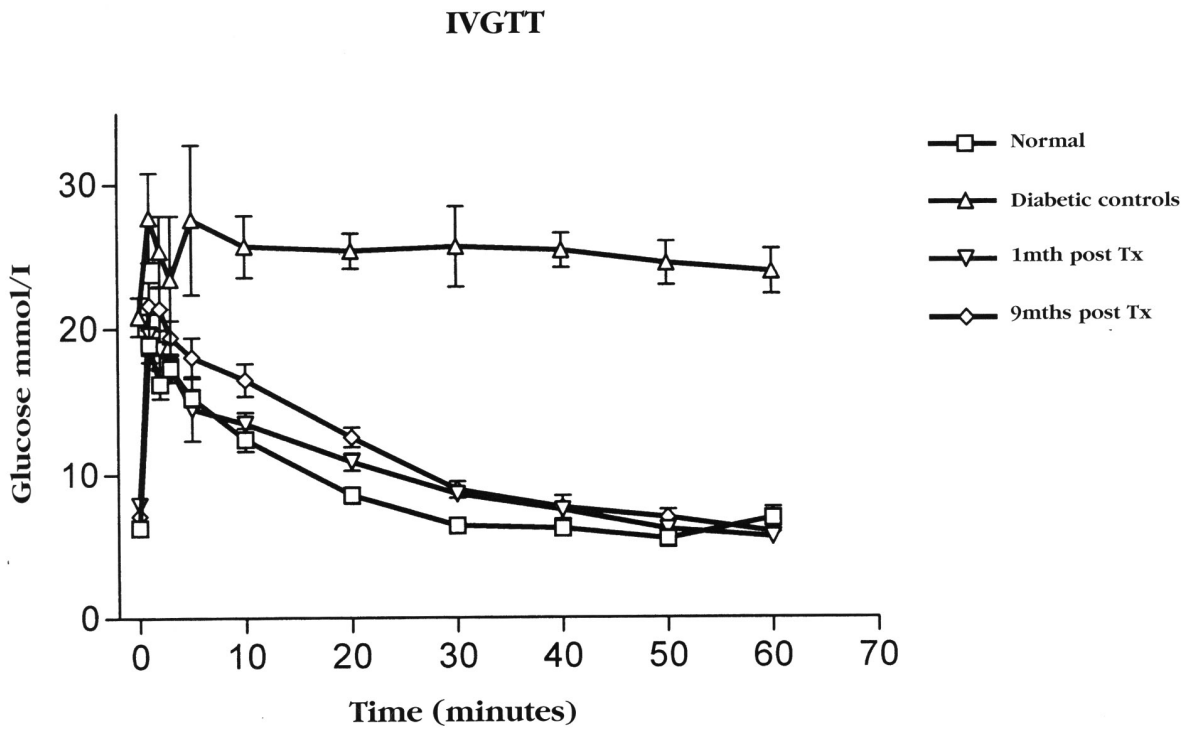

Fig. 1.- IVGTT graph illustrating the different glucose tolerance test values between experimental groups.

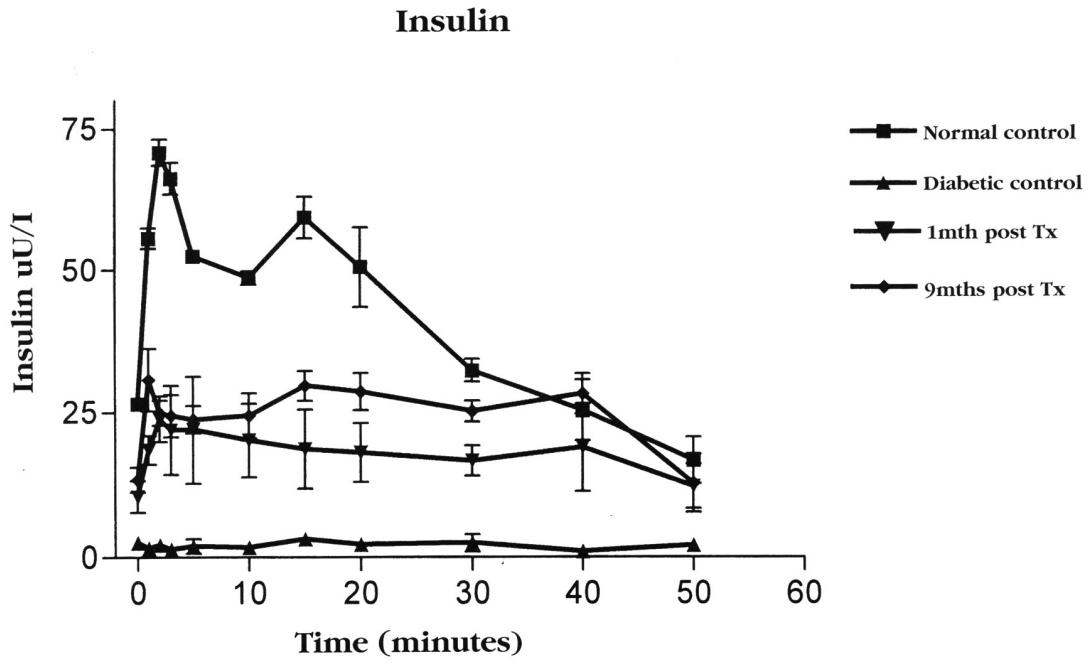

Fig. 2.- Insulin secretion illustrated between the different experimental groups in response to glucose stimulation during IVGTT as determined by coat-a-count® RIA ^{125}I analysis.

Table 4.- Experimental animal weights.

Group	Weights (g)	P values
Control (n =7)	344 ± 12.0	
STZ (n=6)	249 ± 8.0	0.005
Tx 1 month (n=6)	325 ± 12.1	0.4
Tx 9 months (n=4)	410 ± 3.2	0.005

Data values are mean ± SEM.

Table 5.- Metabolic profile of experimental groups.

Group	Water intake ml/24hrs	Urine output ml/24hrs
Normal control (n =7)	21.3 ± 6.8	7.9 ± 1.3
Diabetic control (n=6)	55.8 ± 10.9 Grp 1; p = 0.002	29.9 ± 6.4 Grp 1; p = 0.008
1 month post-Tx (n=6)	19.0 ± 10.7 Grp 1; p = 0.6	7.5 ± 3.4 Grp 1; p = 0.7
9 months post-Tx (n=4)	18.7 ± 9.2 Grp 1; p = 0.8	8.2 ± 5.8 Grp 1; p = 0.4

Post-Tx: post-transplantation. Data values are mean ± SEM.

month post-transplantation, the water intake and urine output had normalized: i.e. water intake 19.0 ± 10.7 ml/24, and urine output 7.5 ± 3.4 ml/24hrs. There was no significant difference compared to normal controls (Grp 3 vs Grp 1; p = 0.6). Glucose, ketones and protein remained absent in the 24hr urine samples.

Whole blood glucose and IVGTT (Table 6 and Fig. 1)

Normal controls (group 1): Normal control animals had a mean non-fasting WBG value of 6.42 ± 0.55mmol/l. IVGTT showed a rapid increase of WBG, from 6.26 ± 0.17 to 18.9mmol/l ± 1.16 range 21.2-12.7mmol/l, one minute after intravenous dextrose injection. A typical response to glucose loading was followed by a return to basal levels after 30 minutes (6.38 ± 0.047 mmol/l). At 50 minutes the WBG level dropped below the basal level (5.45 ± 0.57 mmol/l), followed by slight over-compensation at 60 minutes in some recipients (6.9 ± 0.77 mmol/l). IVGTT glucose clearance rate % (k-value – Lundbaek, 1962) was 2.31 and the area under the curve (AUC) 511.1mm^2.

Diabetic controls (group 2): The animals of the diabetic control group were unable to launch an effective response to the glucose bolus during the IVGTT, with WBG levels of 23.88 ± 1.57 mmol/l, still well above the basal values of 20.84 ± 1.3 mmol/l at 60 minutes. No correlation with the normal controls, r = 0.4739, could be shown.

1 Month post-transplantation (group 3): WBG determined at 1/12 revealed normoglycaemic values 6.37 ± 0.117 mmol/l (Grp 3 vs Grp 1; p = 0.4).

The IVGTT (Fig. 1) showed a return to basal glucose values at 30 minutes after the glucose bolus. AUC values had been restored to 88% (578.8 mm^2) of the normal curve-area (511.1 mm^2) and the k-values were 1.955 (85% normal). The IVGTT correlated very well with the normal controls (Grp 3 vs Grp 1; r = 0.97).

9 Months post-transplantation: The mean WBG was 6.59 ± 0.229 mmol/l (range 6.0-8.1 mmol/l). Comparison of the non-fasting whole blood glucose levels at one and 9 months showed that they were not significantly different (Grp 4 vs Grp 3; p = 0.3).

IVGTT at 9 months showed a return to basal values 40 minutes after the glucose bolus, the near normal curve-area was maintained at 78% (653.4 mm^2) and a k-value of 1.477, 64% of the normal value. Although the IVGTT showed a

Table 6.- Intravenous glucose tolerance test values.

Time	Normal control (mmol/l)	Diabetic control (mmol/l)	1/12 post-Tx (mmol/l)	9/12 post-Tx (mmol/l)
Basal	6.29 ± 0.17	20.84 ± 1.3	7.9 ± 0.13	7.13 ± 0.48
1 min	18.91 ± 1.16	27.7 ± 3.1	19.63 ± 1.39	21.6 ± 1.63
2 min	16.24 ± 0.98	25.36 ± 2.44	18.65 ± 1.29	21.37 ± 1.47
3 min	17.3 ± .7	23.42 ± 4.38	17.35 ± 0.964	19.4 ± 1.19
5 min	15.3 ± 0.58	27.56 ± 5.24	14.5 ± 2.14	18.07 ± 1.30
10 min	12.4 ± 0.81	25.64 ± 2.16	13.5 ± 0.77	16.5 ± 1.10
20 min	8.53 ± 0.57	25.34 ± 1.22	10.85 ± 0.6	12.57 ± 0.67
30 min	6.39 ± 0.47	25.62 ± 2.81	8.58 ± 0.21	8.9 ± 0.57
40 min	6.2 ± 0.48	25.36 ± 1.2	7.47 ± 0.49	7.67 ± 0.79
50 min	5.46 ± 0.57	24.46 ± 1.51	6.15 ± 0.25	6.97 ± 0.52
60 min	6.9 ± 0.77	23.88 ± 1.57	5.6 ± 0.2	5.93 ± 0.32

Post-Tx: post-transplantation. Data values are mean ± SEM.

Table 7.- Intravenous glucose insulin response.

Time	Normal control (μIU/l)	Diabetic control (μIU/l)	1/12 post-Tx (μIU/l)	9/12 post-Tx (μIU/l)
Basal	26.58 ± 0.73	2.54 ± 0.88	10.52 ± 2.75	13.4 ± 2.14
1 min	55.7 ± 0.86	1.66 ± 0.1	18.59 ± 2.46	30.81 ± 5.52
2 min	70.93 ± 1.76	2.17 ± 0.35	23.93 ± 3.95	24.98 ± 2.17
3 min	66.34 ± 1.03	1.42 ± 0.23	22.06 ± 7.73	24.47 ± 3.67
5 min	52.49 ± 1.80	1.94 ± 1.22	22.07 ± 9.33	23.83 ± 2.4
10 min	48.67 ± 1.67	1.75 ± 0.37	20.26 ± 6.39	24.53 ± 3.89
15 min	59.4 ± 2.43	3.18 ± 1.48	18.73 ± 6.84	29.75 ± 2.63
20 min	50.63 ± 1.55	2.22 ± 1.01	18.12 ± 5.12	28.71 ± 3.26
30 min	32.45 ± 1.34	2.49 ± 1.51	16.80 ± 2.58	25.28 ± 1.84
40 min	25.46 ± 1.75	1.01 ± 0.35	19.06 ± 7.6	28.36 ± 3.53
50 min	16.77 ± 1.53	2.13 ± 0.57	12.32 ± 2.72	12.84 ± 2.42
60 min	6.9 ± 0.82	1.91 ± 0.16		

Post-Tx: post-transplantation. Data values are mean ± SEM.

slight deterioration in the glucose clearance (Grp 3; k =1.955 vs Grp 4; k = 1.477), there was still good correlation with the normal controls (Grp 4 vs Grp 1; r = 0.97).

Insulin Response to IVGTT (Table 7 and Fig 2)

Normal controls (group 1): Insulin response to a single glucose bolus showed the typical biphasic insulin graph with a rapid initial insulin response reaching 70.93 μIU/l at 3 minutes. This was followed by a lag phase (48.67 μIU/l at 10 minutes) and a second peak appearing between 10 and 15 minutes (59.4 μIU/l) where after a gradual decline in the insulin levels ensued, passing the basal levels in 30-40 minutes. The mean plasma insulin AUC was 2006 mm^2 following glucose stimulation.

Diabetic controls (group 2): Insulin levels following glucose stimulation remained below 3.18 μIU/l (Sensitivity of kit <4) throughout the test with a mean AUC of 100.9 mm^2 and showed little correlation with normal controls, r = 0.6.

1 Month post-transplantation (group 3): Glucose-stimulated peak serum insulin levels were 23.93 μIU/l at 2 minutes followed by a gradual decline in serum insulin. The AUC was 908.1 mm^2 (45.3% of the normal). The insulin response also correlated well with that of normal controls (Grp 3 vs Grp 1; r = 0.8).

9 Months post-transplantation (group 4): the insulin response graph showed an early rapid response peak of 30.81 μIU/l at one minute, with a lag phase and a secondary response of 29.75 μIU/l after 15 minutes. At 50 minutes, insulin levels had returned to below the basal value. The AUC was 1270 mm^2 (63.3% of normal). The insulin response correlation with the normal controls just failed to reach significance (Grp 4 vs Grp 1; r = 0.6 and p = 0.06). Assessing the AUC in the last 40 minutes i.e. 10 minutes after stimulation: the AUC of the normal control group was 1461 mm^2 compared to 699.4 mm^2 (47.9% of normal) at one month and 1026 mm^2 (70.2% of normal) at 9 months.

Histology

Non-transplanted foetal control tissue: Light-microscopic histological evaluation of control non-transplanted foetal pancreatic tissue 16-18 days gestation showed developing ducts and acinar tissue together with visible islet tissue (at 16 days). The interstitial cells consisted mostly of undifferentiated spindle shaped cells.

Control adult pancreatic tissue (group1): Light microscopy of the control adult rat pancreata showed the typical pancreas architecture, 80-90% of the pancreas consisting of exocrine tissue, which included pancreatic acini and collecting ducts. Discrete islets of Langerhans of between 50-100 μm and some bigger coalesced islets of up to 300 mm in diameter were dispersed throughout the pancreas.

Table 8.- Morfhometric data.

	Normal control (μm^2)	Diabetic control (μm^2)	1/12 post Tx (μm^2)	9/12 post Tx (μm^2)
% β-cell area/islet area (grp vs control)	64.14 ± 1.94	28.4 ± 12.48 (p=0.01)	57.12 ± 6.2 (p=0.3)	52.72 ± 8.06 (p=0.3)
β-cell size (grp vs control)	158.43 ± 8.16	66.25 ± 6.77 (p=0.01)	123.78 ± 20.01 (p=0.2)	144.39 ± 16.89 (p=0.6)
β-cell/α-cell (grp vs control)	2.24 ± 0.16	0.44 ± 0.26 (p=0.01)	17.4 ± 1.9 (p=0.01)	10.25 ± 2.95 (p=0.01)
% α-cell area/islet area (grp vs control)	21.26 ± 1.54	50.7 ± 13.38 (p=0.03)	4.30 ± 8.45 (p=0.01)	11.49 ± 3.4 (p=0.04)
α-cell size (grp vs control)	141.55 ± 10.69	112.99 ± 44.15 (p=0.4)	61.9 ± 10.69 (p=0.01)	84.37 ± 7.26 (p=0.01)

Data values are mean ± SEM.

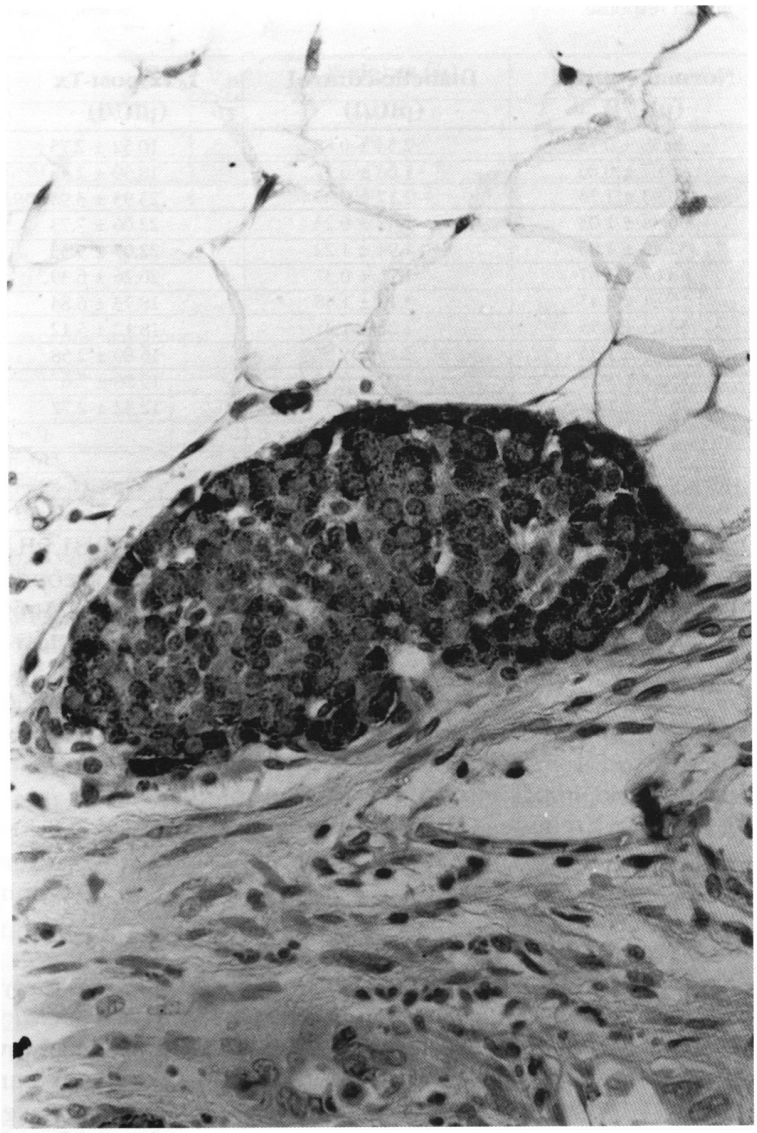

Fig. 3.- Nine months post-transplantation. Well-developed islets of Langerhans consisting mostly of insulin-positive b-cells were present in either fat or fibrous tissue. x 800

Diabetic controls (group 2): islets of Langerhans were present but were small, < 80 μm in diameter. The islet cells appeared to be smaller than seen in the normal controls with nuclei closer together.

1 Month post-transplantation (group 3): Grafts harvested at 30 days showed 3-5 well developed islets of Langerhans per high field magnification (x40). The islets appeared to have developed normally and were 50-100 μm in diameter. The exocrine tissue had almost totally atrophied only occasional pancreatic acini persisting. The collecting ducts appeared dilated and their lumina contained many apoptotic cells.

9 Months post-transplantation (group 4): By 9 months the grafts were completely embedded in white adipose tissue, visible as discrete fat humps on the surface of the kidney, measuring up to 24 x 10 mm in size. All the exocrine tissue had disappeared with only the islets surviving. The islets were either loose in the surrounding adipose tissue or embedded in a fibrous stroma. The islets appeared normal and measured 75-200 μm. Some masses of coalesced islets of up to 600 μm were evident in the fibrous stroma of some specimens.

Immunocytochemistry

Non-transplanted foetal pancreatic control tissue: Immunocytochemistry of foetal pancreatic tissue 16-18 days gestation, to demonstrate insulin and glucagon, showed only faint staining of β- and α-cells. No cells were detected which stained positive for either somatostatin or pancreatic peptide.

Normal adult control pancreatic tissue (group 1): Immunocytochemistry of these islets using antibodies against insulin, glucagon, somatostatin and pancreatic peptide showed a consistent pattern of staining. The insulin-positive staining β-cells were most common (70-80% of islet cells) and occupied the central portion of the islet.

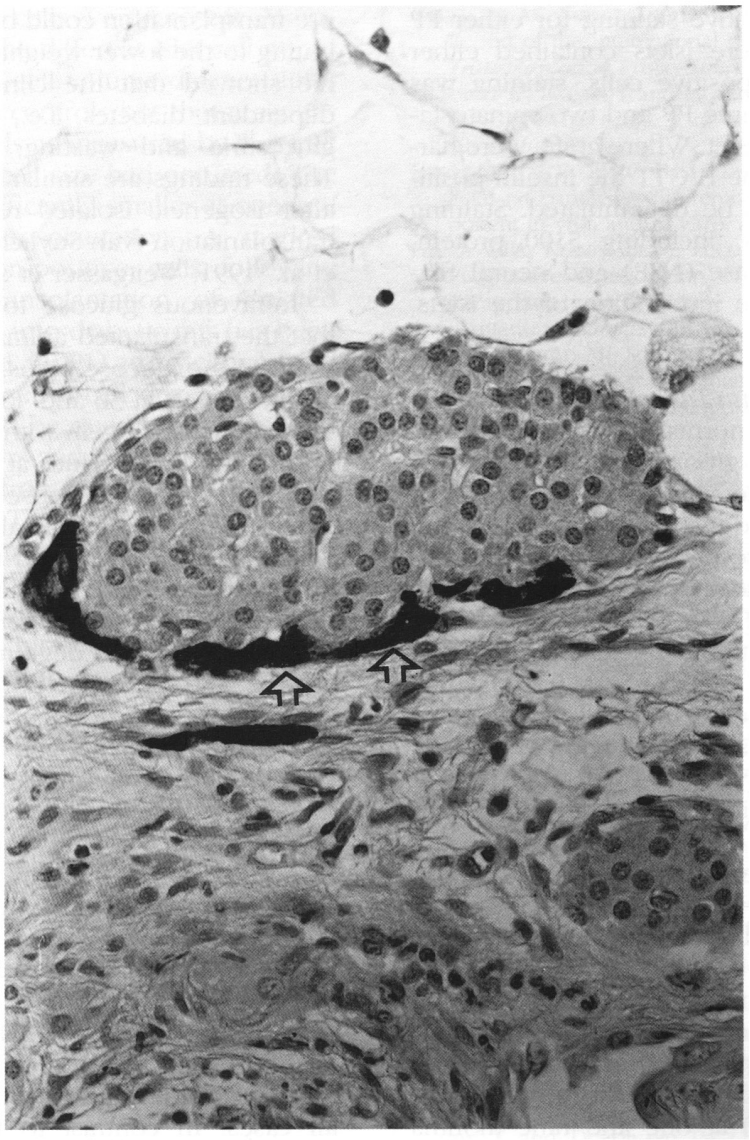

Fig. 4.- Nine months post-transplantation. A thin incomplete band of glucagon-positive staining a-cells limited to the peripheral margin of the islet (arrows) is seen. Morphometry confirmed there were significantly fewer glucagon-positive staining a-cells in the engrafted islets, at 1 and 9 months post-transplantation, compared to normal pancreatic islets. x 800

Glucagon positive staining α-cells were restricted to the peripheral part of the islet and constituted about 15% of the islet cells. A few single δ-Cells, < 18 cells per islet, staining positive for somatostatin, were seen scattered peripherally in the islets. PP-positive staining cells, < 29 cells per islet, were observed in the peripheral part of the islets. The areas in which somatostatin- and PP-positive cells were seen corresponded to the peripheral glucagon-positive staining areas of the islets. A few extra-islet PP-positive cells, < 1 per x40 magnification, associated with the secretory acinar units, were observed scattered throughout the exocrine tissue.

Diabetic control group (group 2): Immunocytochemistry (ICC) showed that the islets consisted almost entirely of glucagons-positive cells. In some of the bigger islets, a diminished central insulin-positive area was still present, but in smaller islets no insulin-positive cells were found. A significant amount of PP-positive cells, (8-27 cells/islet) were scattered either randomly in the small islets or in the peripheral part of the larger islets. Somatostatin staining showed a similar distribution of cells as the PP staining but with fewer, (3-12 positive) cells per islet.

One month post-transplantation (group 3): ICC showed that the islets consisted almost entirely of insulin-positive cells. Glucagon staining cells were limited to a thin interrupted band of cells on the periphery of the islets. A few PP-positive cells (<6 per islet) were still present in the peripheral portion of the islets. Staining for somatostatin revealed fewer than three 3 positive cells per islet.

9 Months post-transplantation (Group 4): Staining for insulin, glucagon, somatostatin and pancreatic peptide showed that islets consisted almost entirely (85-90% of islet cells) of insulin-positive β-cells (Fig. 3). A thin interrupted band of glucagon-positive α-cells (Fig. 4) was present in the peripheral mantle of the islets. Most of the

islets showed no positive staining for either PP or somatostatin. Where islets contained either PP- or somatostatin-positive cells, staining was limited to less than three PP and two somatostatin-positive cells per islet. Where grafts were harvested shortly after the IVGTT the insulin-positive cells appeared to be degranulated. Staining with neural markers, including S100 protein, neuron specific enolase (NSE) and neural filaments to demonstrate innervation of the islets, proved negative.

Morphometric analysis (Table 8)

Compared to the normal controls (Table 8), the diabetic control group had a significantly reduced β-cell volume as measured by the % β-cell area/islet area and the β-cell/α-cell ratio ($p = 0.01$ and $p = 0.01$ respectively). Remaining β-cells were also significantly smaller compared to the controls ($p = 0.01$). The % α-cell volume per islet as calculated by the % α-cells area/islet area showed a significant increase ($p = 0.03$), which was due to the decrease in β-cells induced by steptozotocin in the islets.

Comparing β-cell volume of the grafts, at 1 and 9 months, the differences were not significantly different on comparing the % β-cell area/islet area or β-cell size ($p=0.03$, $p=0.02$ at 1 month and $p=0.03$, $p=0.06$ at 9 months, respectively). The β-cell/α-cell ratio was, however, significantly increased ($p=0.01$) due to a reduction of α-cell volume, as measured by the % α-cell area/islet area and α-cell size. The α-cells of the grafts were also significantly smaller (Grp 3; 61.9μm^2, $p = 0.01$ and Grp 4; 84.37μm^2, $p = 0.01$ vs grp 1; 141.55μm^2) at one and nine months post-transplantation compared to normal controls.

Discussion

Intravenous injection with 50 mg/kg streptozotocin results in a permanent and rapid onset of type 1 diabetes (Bouwens et al., 1996; Steiner et al., 1970).

Renal subcapsular foetal pancreas transplantation is capable of normalizing the gross clinical and metabolic aberrations of streptozotocin-induced diabetes mellitus in a laboratory rat model and succeeds in maintaining a near normal non-fasting blood glucose level both in the short- and long-term. At one month and nine months after the restoration of normoglycaemia by foetal pancreas transplantation, non-fasting whole blood glucose levels were in the range of normal controls. Although the rats gained weight and appeared to grow normally after transplantation they failed to reach the weight of the normal age-matched controls. Streptozotocin toxicity and the metabolic deficiencies of diabetes pre-transplantation could be major factors contributing to the lower weights. The metabolic profile showed that the clinical signs of insulin-dependent diabetes, i.e., polydipsia, polyuria, glucosuria and wasting, had been reversed. These findings are similar to those documented after isogeneic isolated renal subcapsular islet transplantation (van Suylichem et al., 1992; Hiller et al., 1991; Weitgasser et al., 1995).

Intravenous glucose tolerance tests showed that the transplanted animals where able to respond to the glucose bolus, returning to baseline glucose levels at 30 and 40 minutes respectively. This compared well with the controls, which returned to basal values at 30 minutes. Comparison of the control glucose clearance rate to that of the transplanted animals showed that the k-values had been restored to 85% at one month but had deteriorated to 64% of normal at nine months post-transplantation. This may be due to islet attrition, β-cell degranulation, reduced islet reserve and peri-islet fibrosis. Analysis of the area under the IVGTT-curve showed that glucose metabolism had been restored to 88% and 78% of normal at one and nine months post-transplantation, respectively. Although both the k-values and the area under the IVGTT-curve showed a subnormal response to the glucose bolus, it must be acknowledged that the IVGTT is far beyond the normal dietary carbohydrate load and therefore probably reflects the reserve capacity rather than the normal physiological requirement (Hiller et al., 1991).

The insulin response of the transplanted animals was significantly lower than the controls in all cases. In contrast to the IVGTT, the area under the curve showed that the 9 months post-transplantation group gave a better insulin response, and showed signs of the biphasic response seen in the controls, but which was absent at one month post-transplantation.

Histologically, the grafts showed a classic development, the exocrine tissue being replaced with fibrous tissue at one month (Du Toit et al., 1997; Feldman et al., 1980). The islets were well developed within this fibrous stroma. As previously reported by Du Toit el al. (1997) and McEnvoy and Hegre (1979), by nine months the grafts were completely encapsulated in white adipose tissue with a large proportion of the islets lying freely within the peri-graft fat while some islets were still entrapped within the fibrous stroma. The greater vascularity within the adipose tissue might explain the improved insulin secretion seen in these older rats. Immunocytochemistry of the engrafted islets at one and nine months post-transplantation showed that most of the islet consisted of insulin-positive β-cells. Glucagon positive α-cells were limited to a thin discontinuous band on the peripheral margin of the islets while only occasional somatos-

tatin- and PP-positive cells were present. Morphological analysis of the different islet cell populations showed that β-cell volume did not differ significantly from the controls but that a significant decrease in α-cells as measured by % α-cell area/islet area and α-cell size had occurred. The α-cells were also significantly smaller at one and nine months post-transplantation. A marked reduction in glucagon-positive cells following renal subcapsular transplantation of isolated islets and foetal tissue into diabetic rats has been reported by Hiller et al. (1991) and McEnvoy and Hegre (1979). Decreased α-cell development in the grafted islets could be due to an absence of exocrine growth factors and possibly some form of negative feedback from functional α-cells present in the pancreas. The effect of fewer glucagon positive staining α-cells on endocrine function within the transplanted islets is not clear. Denervation of the islets, especially parasympathetic innervation, is a further aberration complicating endocrine secretion.

In conclusion, engraftment of foetal pancreas tissue under the kidney capsule results in the development of functional and active islets of Langerhans, capable of restoring normoglycaemia and reversing the clinical signs of streptozotocin-induced type 1 diabetes in this laboratory diabetic rat model.

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