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Immunomodulation Techniques in a Pig-to-Rat Model of Xenoislet Transplantation to Prolong Graft Survival*

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Abstract. *Pancreas allotransplantation can restore full metabolic control in patients with type I diabetes, but has several limitations. Pancreatic islet xenotransplantation (XiTx) is considered a reliable alternative. The aim of this study was to evaluate the effect of gamma-irradiation and a highly selective inducible nitric oxide synthase inhibitor (AE-ITU) in a model of pig-to-rat XiTx. Thirty-five female rats were made diabetic by intraperitoneal injection of streptozocin. Pig pancreatic islets were obtained by enzymatic digestion followed by purification on Ficoll gradients. Approximately 4,000 purified pig islet equivalents were placed under the left kidney capsule of the recipient rats. The rats were observed for 15 days and divided into five Groups (G): G1: controls, diabetic rats with no treatment; G2: XiTx; G3: XiTx after gamma-irradiation (20 Gy); G4: XiTx and administration of AE-ITU; G5: XiTx after gamma-irradiation and AE-ITU. Graft survival was defined as the maintenance of the glucose levels at less than 11 mmol/l and a normal response to i.v. glucose challenge. The graft survivals in Groups 2, 3, 4 and 5 were 4.1±1.8, 7.6±2.1, 7.6±2.4, and 10.9±2.3 days, respectively. The graft survival of G2 was significantly ($p<0.05$) lower than the other groups, and the graft survival of G5 was significantly higher in respect of both G3 and G4 (log-rank test: $p=0.007$). In conclusion, the combination of AE-ITU (to reduce the early inflammatory damage) and gamma-irradiation (to reduce the immunogenicity of the islets) may be considered an interesting option to prolong the euglycaemic period after XiTx.*

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Insulin-dependent (type I) diabetes mellitus (IDDM) is a common disease, with many severe long-term complications (1). Whole pancreas allotransplantation can restore full metabolic control in IDDM, but has many limitations, since it entails major surgery and long-term immunosuppression (2). Islets transplantation is a viable and attractive option, but the shortage of human donors is still a problem (3). Islet xenotransplantation (XiTx) has been widely applied in the experimental setting, but the reduced immunosuppression-free survival of the cells still remains the major obstacle to successful clinical application (1, 2, 4). Different strategies have been used, such as immunosuppression, immuno-modulation, immuno-isolation and methods of tolerance induction (5). More recently, the crucial role of nitric oxide (NO) in the immune response and allograft rejection has been proved and the use of selective inhibitors of inducible nitric oxide synthase (iNOS) has become feasible (6, 7). The aim of this study was to evaluate the effect of the inhibition of NO synthesis with or without an immuno-modulation technique (gamma-irradiation) on xenograft survival in a model of XiTx in rats.

Materials and Methods

Animal studies were performed in compliance with the International Guidelines for humane care of experimental animals. Pig islets were isolated from young male Yorkshire pigs, weighting 25-30 kg, with an enzymatic digestion procedure (8). All operations were performed under general anaesthesia (20 mg/kg, *i.v.* ketamine, and 0.5-1.0% endotracheal isoflurane) using a sterile surgical technique. Islets were obtained from a collagenase (Sigma, St. Louis, MO, USA) digested pancreas on Ficoll gradients, cultured in RPMI 1640 media (Gibco, Grand Island, NY, USA), supplemented with 20% pig serum, 10 mM HEPES (Gibco) and antibiotics (streptomycin sulfate 0.1 mg/ml, penicillin 100 IU/ml, amphoterycin B 2.5 μg/ml) at 37˚C, 5% CO_2 (9). Islets viability was assessed by trypan-blue exclusion test. Thirty-five Sprague Dawley female rats, weighting 200 to 250 g, were made diabetic by intraperitoneal streptozocin (STZ) injection (90 mg/kg body weight), under ether anaesthesia. Non-fasting glycaemia increased two days after STZ injection, reaching more than 22 mmol/l in all

Table I. *Graft survival (days, mean±SD) and main biochemical parameters in each Group.*

Group	Surv	Gly	AGT	Pro	Mic
G1	-	7/7	7/7	2/7	3/7
G2	4.1±1.8	2/7	3/7	0/7	1/7
G3	7.6±2.1	0/7	1/7	0/7	0/7
G4	7.6±2.4	0/7	2/7	0/7	0/7
G5	10.9±2.3	0/7	0/7	0/7	0/7

Surv=survival, Gly=glycosuria, AGT=abnormal glucose challenge test, Pro=proteinuria, Mic=microhaematuria.

recipients 4-5 days after STZ administration. This level was considered reliable for experimental significance (10). The rats were divided into five groups (G1 to G2, 7 animals each) and observed for 15 days. G1: controls, diabetic rats with no treatment; G2: XiTx; G3: XiTx after γ-irradiation (20 Gy); G4: XiTx and administration of S-2-aminoethyl-isothiourea (AE-ITU), a selective inhibitor of iNOS; G5: XiTx after γ-irradiation and AE-ITU administration. Blood samples were drawn daily from the tail vein. Non-fasting blood glucose levels (GL) <11 mmol/l were defined as normoglycaemic. Graft survival was defined as the maintenance of GL at less than 11 mmol/l and a normal response to *i.v.* glucose challenge (performed 3 days after XiTx). Two subsequent GL exceeding 11 mmol/l were considered as a graft failure. To exclude the influence of kidney damage from the XiTx procedures, a urine analysis test was performed on recipients 5 days after XiTx. The islet transplantation was performed through a midline incision under ether anaesthesia and ketamine administration (20 mg/kg). Approximately 4,000 purified pig islets, in a minimal volume of Hank's balanced salt solution, were placed under the left kidney capsule utilizing a Hamilton syringe, 5 days after STZ injection. Islets were irradiated in culture medium in Falcon tubes. The dose of irradiation was 20 Gy, considered the limit to avoid damage to function of the beta-cells (11). AE-ITU was administrated intraperitoneally every 12 hours for 7 days, at a dosage of 50 mg/kg/day, diluted in 0.5 ml of saline solution, adjusted to pH 3 using citrate buffer, to prevent spontaneous conversion of aminoalkylisothioureas into mercaptoalkylguanidines (12). The surviving rats were sacrificed 15 days after XiTx, evaluation of the abdomen and peritoneum was conducted, and histological examination of the left kidney was performed. All values are expressed as mean ± standard deviation (SD). Overall survival was calculated according to the Kaplan-Meier method, and differences in survival between Groups were evaluated with the log-rank test. The significance level was set at $p<0.05$. All analyses were performed using Statistica 6.0 package (StatSoft, Tulsa, OK, USA).

Results

The mean graft survival and the main biochemical parameters in each Group are reported in Table I. *G1*: No reduction of blood glucose levels was observed. Histological examination showed moderate tubular lesions of the kidney in three cases. *G2*: XiTx restored euglycaemia in 1-2 days. In two animals the euglycaemia lasted only 2 days. Histological and immuno-histochemical examinations of the left kidney showed massive cellular infiltrate, prevalently macrophage-like cells, and no morphologically intact endocrine cells were found. *G3*: Histological signs of mild tubular lesions of the kidney were found in two cases. *G4*: Light tubular lesions of the kidney were found in two cases. *G5*: No alterations of the kidney or other organs were found. The graft survival of G2 was significantly ($p<0.05$) lower than the other Groups, and the graft survival of G5 was significantly higher in respect of both G3 and G4 (log-rank test: $p=0.007$), while no difference was found (p=NS) between G3 and G4 (Table II). The median survival was 5 days (95% CI: 2-na) in the control Group, 8 days (95% CI: 7-9 days) in the Groups 3 and 4, and 10 days (95% CI: 9-na) in the Group 5. The cumulated proportions of survival according to the Kaplan-Meier method are shown in Figure 1.

Table II. *Log-rank test between Groups (G) and relative p-value.*

	G3	G4	G5
G2	0.1	0.03	0.006
G3	-	0.84	0.02
G4		-	0.04

Discussion

Production of NO is one of the most important modalities by which activated macrophages damage cells and tissues, such as xenogenic and allogenic islets, and iNOS were found in induced macrophages (13). Massive infiltration of macrophage-like cells, found also in our autopsic examination, can be considered the cause of β-cells dysfunction. Substances which are able to inhibit NOS have been shown to limit the damage to β-cells (14, 15). We have previously found that aminoguanidine prolonged the survival of islet xenografts in diabetic recipients, but its selectivity for iNOS is only partial and its inhibitory activity is very weak (16, 17). To protect islets from immune attack, several immunotherapeutic modalities have been proposed (1-5, 18, 19). Gamma-irradiation was considered effective in elimination of donor dendritic cells and of residual, highly antigenic, exocrine cells that had been spared by purification techniques (20, 21). We irradiated (20 Gy) the pancreatic cells prior to transplantation with the highest dose compatible with maintenance of β-cell function (22). In our study, no significant (p=NS) differences were found between survival of G3 and G4, and, thus, the two immunomodulation techniques were similarly effective. However, using the combination of gamma-irradiation and AE-ITU

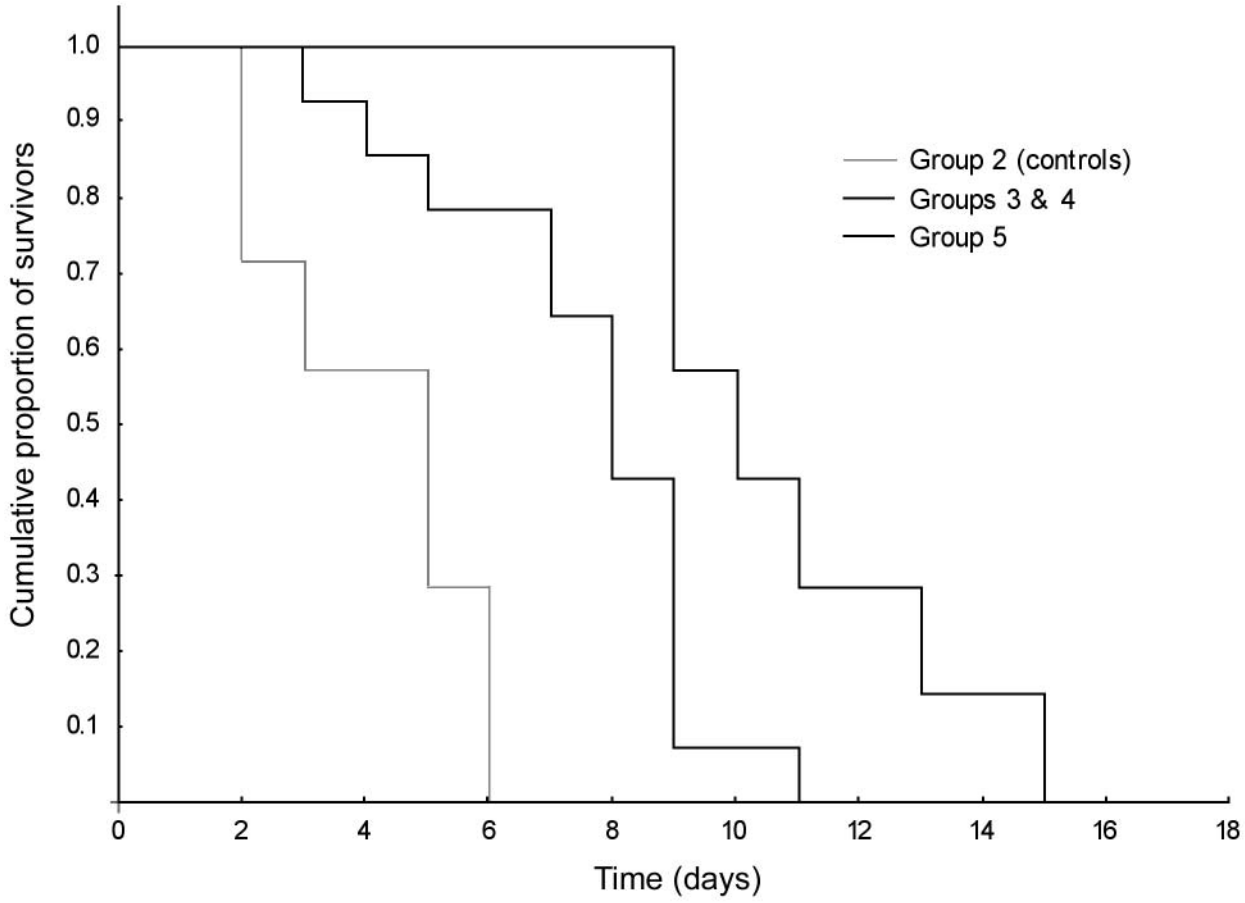

Figure 1. *Cumulated proportion of survival according to Kaplan-Meier method.*

administration, the mean graft survival improved significantly. The rate of abnormal GCT decreased in treated Groups, emphasizing either the longer survival, or the better function, in terms of response to glucose stimulus of the engrafted cells. The combination of an inhibitor of iNOS (to reduce the early inflammatory damage of the graft) and gamma-irradiation (to reduce the immunogenicity of the islets) may be considered an interesting option. We have confirmed that the combination of gamma-irradiation and AE-ITU significantly prolonged the euglycaemic period in diabetic recipients, even though the results had been expected to be more impressive. The effects were not synergistic, maybe because both the target and the timing of the two immunotherapeutic modalities differed.

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