FACTORS INFLUENCING THE EARLY RESULTS OF PANCREATIC ISLET TRANSPLANTATION: STUDIES ON ISOLATION ENZYMES AND ISLET REVASCULARISATION

Ph.D. theses

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Budapest
2007.
1. INTRODUCTION

Diabetes mellitus, affecting approximately 170 million people in the world is regarded as an endemic disease in the developed industrial countries. Compared to the normal population, blindness is twenty-five times, chronic kidney failure is seventeen times, cardiovascular diseases are twice more frequent among the patients suffering from I-type diabetes. 34% of the patients develop an end stage kidney failure within 15 years, while in 10% of them amputation has to be done due to severe angiopathy. Diabetic retinopathy is the main cause of blindness in the age-group between 20 and 60. Cardiovascular diseases resulted by the advanced atherosclerosis present the main cause of death in the diabetic population. Their life expectancy - without an appropriate treatment- is about two thirds of that of the normal population. A significant increase of the number of diabetic patients is expected in the forthcoming years; according to a WHO forecast, their number can approximate 366 million by 2030. Considering the rapid growth of the number of patient, research for new therapeutic methods is necessary.

Despite the intensive researches done all over the world the treatment of diabetes is not settled even today. Recent clinical studies support the suggestion that the only way to regain the physiological regulation of carbo-hydrate metabolism is the transplantation of the insulin-producing islets of Langerhans. One form of that is the independent transplantation of islets isolated from the pancreas, promisingly developing since the turn of the century. That is due to the elaboration and introduction of the Edmonton protocol changing the guidelines of patient selection; to the new immunosuppressive drugs introduced; and to the international co-operations launched. Mass application of the method is still hindered by the fact that separation of islets of implantable quality is possible only in the 50-60% of the isolations, besides, a considerable loss - sometimes affecting the 40-50% of the islets - is observed during the early phase of the transplantations. Thus, a sequential transplantation of islets, beyond the amount of the physiological demand and collected from 2-3 braindead donors is necessary for one diabetic patient. The global shortage of donors presses for a further analysis of the two above-mentioned problems and for the development of new, alternative methods.

One of the central questions of human islet isolation is the variability and unpredictability of the enzymes required for the digestion of the pancreas tissue. Introduction of an enzyme with a controlled composition and with a more accurate dosing may enhance the efficacy of the
isolations and may improve the vitality of the separated islets. In the first part of the present thesis I am going to study one of these new enzyme products.

Recent studies drew attention to the fact that the complex vascular system of the Langerhans islets is damaged during the enzymatic isolation process and the insufficient vascularisation may be one of the main factors of the early islet cell loss observed following transplantation. The islets have a well-developed microvascular reticular system and their appropriate function after transplantation requires the regeneration of this complex vascular system. Thus, study of the revascularisation enhancing factors may contribute to the reduction of the number of islets to be transplanted and to their improved functioning.

Data of recent publications indicate that VEGF-A /vascular endothelial growth factor/, regarded as an important agent in tumour angiogenesis, has a key role in the revascularisation of the transplanted islet cells as well. It has been suggested that angiogenesis around the islets could be improved and the loss of islets could be decreased through an increase of local VEGF-A production. In the second part of the thesis I am studying how the revascularisation and function of the transplanted islets is affected by the increased VEGF-A expression and whether it can be regulated by a pharmacological method.
2. OBJECTIVES

The aim of both projects used as a base for the present doctoral thesis is to improve the efficacy of human islet transplantations and to optimise the post-transplantation revascularisation in order to decrease the early islet cell loss. In the first part of the thesis I am studying a new human isolation enzyme product, while in the second part I am analysing the effects of an increased VEGF expression on revascularisation and endocrine functions of islets following transplantation in an animal experiment. Considering the potentially negative effects of an uncontrolled VEGF secretion, I also intended to elaborate in vitro and in vivo models capable of pharmacologically regulating VEGF expression and consequently the islet cell vascularisation.

During the enzyme studies I was asking the following questions:

1. How stable are the different lots of Collagenase NB1/Neutral Protease NB1 enzymes with regard of digestive activity in comparison to Liberase HI enzymes?

2. Is there any difference in islet yield, morphology and apoptosis of islets isolated with the two enzymes?

3. Does it differ the glucose stimulated in vitro insulin secretion of islets isolated with the two enzymes?

4. How are functioning the islets isolated with the two enzymes in an in vivo animal model and what are the preliminary results following human transplantation?

During the VEGF experiments I was studying the following questions:

5. Does the VEGF-A overexpression has any influence on the in vitro glucose stimulated insulin secretion of isolated RIP-VEGF transgenic mouse islets?

6. Can the transplantation of islets isolated from RIP-VEGF homozygous transgenic mice regulate the carbo-hydrate metabolism of diabetic recipients?

7. Could the genetically engineered CDM3D-TET-VEGF beta-cell line have a stable in vitro VEGF secretion?
8. How does it influence the VEGF-A expression of CDM3D-TET-VEGF beta-cells the in vitro angiogenesis if co-cultured with endothelial cells?

9. Could this in vitro process be regulated by adding Tetracycline to the system?

10. Does the genetic modification have any influence on glucose stimulated in vitro insulin secretion of the modified CDM3D-TET-VEGF beta-cells?

11. How are functioning the CDM3D-TET-VEGF beta-cells following transplantation into syngeneic diabetic mice?

12. How will the VEGF-A overexpression affect the revascularisation and endocrine function of transplanted CDM3D-TET-VEGF beta-cells?

13. Can the locally increased expression of VEGF-A and consecutively the islet revascularisation be regulated with Tetracycline after transplantation?
3. MATERIALS AND METHODS

3.1. Comparative studies with isolation enzymes

Nine consecutive human islet isolations were performed with Collagenase NB1 supplemented with Neutral Protease NB1 (Serva-Electrophoresis GmbH, Heidelberg, Germany) (Group I). These isolations were compared to 9 consecutive human islet isolations performed with Liberase HI (Roche-Boehringer-Mannheim, IN; US) enzymes (Group II). The pancreata were procured from brain-death cadaveric donors in different hospitals and were preserved in 4°C University of Wisconsin solution under aseptic conditions. Islet isolation was performed with a technique routinely used in the Laboratory of Islet Isolation and Transplantation from University of Geneva, and modified from the semi-automated method developed by Ricordi et al. Briefly, the pancreas was perfused with cold enzyme solution (300 mL), using controlled pump perfusion. Digestion was conducted at 37 °C in a digestion chamber. After tissue collection, islets were purified in continuous Biocoll gradients (Biochrom KG, Berlin, Germany), using a COBE 2991 cell processor (Cobe, Lakewood, CO, US). All islet isolations were performed by the same team.

Results were analyzed in terms of islet and islet equivalent (IEQ) yields, tissue volume and purity and islet morphology and viability. Islet and IEQ counts, purity and morphology were assessed by light microscopy. Islet viability was determined by fluorescence microscopy (Zeiss Axiophot, Felbach, Switzerland) after staining with fluorescein diacetate and propidium iodide (Sigma-Aldrich, Gillingham, UK). All assessments were done independently by two observers on two separate samples of the final islet preparation. Islet cell apoptosis was assessed using the Cell Death Detection ELISA PLUS kit (Roche-Boehringer-Mannheim, Germany) which detects nucleosomal particles in cytoplasmic fractions (cell lysates). Islet cell apoptosis analysis was performed on lysates from 100 IEQ, collected after a 12-hour culture. Results are expressed as an enrichment factor in oligonucleosomes of the culture medium over a control medium incubated without islets.

Glucose-stimulated insulin secretion was analyzed in static incubation assays on aliquots of 100 IEQ for each enzyme preparation after a 24-hour culture. Briefly, islets were exposed for 1 hour to the following glucose concentrations: 2.8 mmol/L (basal), 25 mmol/L (stimulated) and 2.8 mmol/L. After each incubation period, supernatants were collected for analysis and islets were washed three times with the next glucose solution. Supernatants were stored at –20 °C before assay. Insulin concentration was measured using a commercially available kit.
Athymic nude mice were rendered diabetic by a single intraperitoneal injection of 200 mg/kg streptozotocin (Sigma, Buchs, Switzerland). Blood sugars levels were monitored on whole-blood samples collected from tail vein. Three aliquots of 1000 human IEQ were transplanted under the left kidney capsule of recipient mice from each islet preparation assessed. Four islet preparations were assessed in each group. Successful islet transplantation was defined by blood glucose levels less than 11 mmol/L on 2 consecutive days.

Nine patients were transplanted with islet preparations obtained in the course of this study. All patients were transplanted intraportally using a percutaneous approach as previously described. Islet preparations were attributed according to priorities unrelated to the present study. In vivo function of the islet preparations was assessed by comparing daily insulin requirements immediately prior to transplantation with daily insulin requirements one month after transplantation.

3.2. Studies on RIP-VEGF islets

The RIP-VEGF transgenic mice are characterized by secreting human VEGF in a Rat Insulin Promoter (RIP) controlled way. In our study we used homozygous transgenic mice. Islets were isolated by means of a previously described technique using a collagenase digestion method. Briefly, islet donor mice were killed by cervical dislocation after an intraperitoneal injection of sodium pentobarbital. The abdomen was opened, the pancreas was exposed and injected with the enzyme solution -collagenase type V, (Sigma Chemical Co, St. Louis, MO, US), 2mg/7ml in Hank’s balanced salt solution (HBSS)- through the main bile duct until full distension was achieved. The pancreatic tissue was then surgically removed and immersed in the enzyme solution. The collagenase digestion was performed in a 18 minute incubation at 37°C, followed by a brief, vigorous, manual shaking, after which enzyme kinetics were sharply slowed by addition of cold HBSS. The tissue was then filtered through a 450 μm screen. Islet purification was obtained by centrifugation at 800 g for 16 min. on discontinuous Euro-Ficoll gradients, and routinely provided islets of purity >90%. Islet purity was assessed
by dithizone (Sigma Chemical Co, St. Louis, MO, US) staining, and the islets were counted and scored for size. An algorithm was used for the calculation of 150 µm diameter islet equivalent number (IEQ, as described before.

Glucose stimulated insulin release was evaluated from isolated islets in vitro. After overnight culture, 50 handpicked islets from RIP-VEGF-A Tg-mice and negative littermates were rinsed with Krebs-Ringer bicarbonate medium supplemented with 0.1% BSA and 2.8mmol/l glucose (control KRB) and pre-incubated in 1ml control KRB for 1 h., at 37°C. Then they were submitted to three successive 1 hour incubations at 37°C; first in 1ml fresh control KRB, then in 1ml KRB containing 16.7mmol/l glucose and then were re-incubated again in 1ml control KRB. The supernatant was stored at -20°C until analyzed by mouse insulin ELISA (Mercodia, Sweden). The samples were assayed in triplicates.

Isolated islets were handpicked in aliquots of 100-150 islets, each under a dissection microscope, and assessed for islet diameter to calculate IEN. The number was adjusted in each aliquot, according to calculated IEN and weight of the recipient, in order to obtain grafts of 3500 IEN/kg body weight. Islets were transplanted under the kidney capsule as previously described. Non-fasting random blood glucose levels were measured daily after transplantation on whole-blood samples in mice transplanted with marginal islet mass. Graft function was defined as occurring on the first of 5 consecutive days of non-fasting blood glucose of 11 mmol/l. Time to normoglycemia was recorded as the primary end point of the experiment.

3.3. Studies on CDM3D-TET-VEGF beta-cells

CDM3D cells are conditionally immortalized mouse beta-cells, which have been engineered to overexpress human bcl-2. They were derived from the βTC-tet cell line, originally isolated by Dr. S. Efrat (Albert Einstein college, NY, US).

Here we have explored the role of temporal VEGF expression, controlled by a tetracycline (TC)-regulated promoter, on revascularization and engraftment of genetically-modified beta-cells following transplantation. To this end, we modified the CDM3D beta-cell line using a lentiviral vector to promote secretion of VEGF-A in a TC-regulated (CDM3D-TET-VEGF-cells). VEGF secretion, angiogenesis, cell proliferation and stimulated insulin secretion were assessed in vitro. The angiogenic activity of VEGF-secreting CDM3D cells was assessed in vitro using a three-dimensional type I collagen gel model that assays both for endothelial cell invasion and capillary-like tube formation.
Three-dimensional collagen gels were prepared in 16mm² tissue culture wells of 4-well plates (Nunc, Roskilde, Denmark) as previously described. Eight volumes of a cold solution of rat tail tendon-derived collagen I (at approximately 1.5mg/ml) were mixed with 1 volume of 10x minimal essential medium (MEM) and 1 volume of sodium bicarbonate (11.76mg/ml) on ice. This mixture was quickly dispensed into tissue culture wells and allowed to gel at 37ºC for 10 minutes. For co-cultures CDM3D or CDM3D-TET-VEGF cells were seeded in suspension at 10'000 or 35'000 cells/well in a 350μl collagen gel. This gel was overlaid with a second, 250μl cell-free layer of collagen. BME cells were subsequently seeded on top of the second layer of collagen at 100'000 cells/well in 500μl of α-MEM/DMEM medium at a ratio of 1:1 together with 5% DCS. Medium was renewed once after 2-3 days, and cultures were fixed after a total of 5 days. In some experiments, a neutralizing antibody to recombinant mouse VEGF 164 (R&D Systems Europe Ltd., Abingdon, UK) was added to the culture medium at a final concentration of 2μg/ml. Invasion was quantified from three randomly selected 1.0 x 1.4 mm fields per well, by measuring the total length of all BME cells that had penetrated into the underlying gel either as single cells or in the form of cell cords. Results are from at least 3 wells per condition from at least two separate experiments.

We used a syngeneic mouse model of transplantation to assess the effects of this controlled VEGF expression in vivo. Time to normoglycemia, intra-peritoneal glucose tolerance test, graft vascular density and cellular mass were evaluated.

Two groups of STZ-induced diabetic syngeneic C3H mice were transplanted under the kidney capsule with cell clusters of either CDM3D or CDM3D-TET-VEGF cells. Two other groups of mice were grafted with each cell type (CDM3D, CDM3D-TET-VEGF) treated in vitro with TC (1µg/ml) for 48 hours, and the drug (1mg/ml) was administered continuously via drinking water to the grafted mice. Two separate groups of mice were transplanted with control and CDM3D-TET-VEGF cells and treated with TC only after the glycemia was corrected (BG<11 mmol/l). Time to normoglycemia and intraperitoneal glucose tolerance test (IPGTT) on day 28 after transplantation were assayed. Six animals were assessed in each groups.

Statistical analysis was performed using the SPSS 10.0 program (SPSS Institute, Chicago, IL, US). Values are expressed as mean ± s.e.m. or as mean ± s.d., as indicated. Comparisons were performed by two-tailed unpaired Student’s t test or by one-way ANOVA. Statistical significance was defined as p < 0.05.
All experiments were performed at the University of Geneva Medical Center and were approved by the institutional Ethical, Animal Care and Use Committee.
4. RESULTS

1. Our data suggests that the lots of CollagenaseNB1/Neutral Protease NB1 enzymes are more stable with regard of activity, there are less lot-to-lot variability in comparison with Liberase HI enzymes. In all isolations (100%, 9/9) of group I, more than 250x10^3 IEQ were recovered, while this was achieved in only 56% (5/9) of procedures in group II (p<0.05). IEQ number per gram of pancreas was statistically significantly higher in group I (4020 ± 1240) as compared to group II (2360 ± 1350; p<0.05). IEQ number to islet number ratio, which reflects the average size of isolated islets, was statistically significantly higher in group I (p<0.01). Final islet preparation volumes were similar in both groups, while purity was higher in group I (p<0.05).

2. Islet morphology was significantly better preserved after isolation in group I as compared to group II. A higher percentage of free and intact islets were recovered in group I (71 ±9%) as compared to group II (52 ±14%; p<0.01). A higher rate of fragmented islets were observed in group II (38 ±14%) as compared to group I (14 ±11%), while the rate of embedded/trapped islet was similar in both groups. Islet cell viability was also similar in both groups, however the purity of final preparations was better in group I (62%±6% ill. 53 %±8%, p<0,05). The rate of islet cell apoptosis was statistically significantly lower in group I, as assayed by intracytoplasmic detection of oligo-nucleosomes within islet cells after a 12-hour culture (1,25%±0,03 ill. 7,25%±1,23, p<0,05). There was no significant difference regarding the donor data, although the mean warm ischemic time was longer in the first group.

3. Insulin release by isolated human islets in response to glucose stimulation was measured in static incubation assays. Stimulation index (SI) was similar in both groups, whereas the acute insulin response (AIR) was higher in group I (p<0.01).

4. In vivo islet function was assessed by transplantation of human islets (1000 IEQ) into chemically diabetic nude mice. All mice from each group reversed diabetes within 48 hours after transplantation. Time to reach normoglycemia was 1.2±0.4 days in group I versus 1.1±0.3 in group II. (p=0.28, NS).
Five islet preparations were transplanted from nine isolations into patients with type 1 diabetes in group I (56%), while four were transplanted in group II (44%). Insulin requirements decreased by more than 50% in all patient transplanted with preparations from
group I, while two patients in group II showed only minimal decrease in their daily insulin dose. Mean insulin requirements before transplantation were 38 ±10 U/day and 50 ±11 U/day in groups I and II, respectively. One month after transplantation, insulin requirement dropped to 8 ±3 U/day and 32 ±13 U/day in groups I and II respectively. Differences in insulin requirement drop were not significant. Two patients in Group I and 1 patient in Group II have become insulin independent by one month after islet infusion. Regarding the small number of cases complimentary data are necessary for further conclusions.

5. Our results from experiments with islets isolated from RIP-VEGF transgenic mice indicates that genetic upregulation of VEGF-A in beta cells did not influence significantly the basal or glucose-stimulated in vitro insulin secretion of islets when compared with control islets.

6. When islets from negative littermates were transplanted, 50% of mice returned to normoglycemia after 26 days and 100% after 39 days. When isolated islets from homozygous RIP-VEGF-A transgenic mice were used, the time to normoglycemia was significantly (P=0.008) reduced: 50% of mice returned to normoglycemia after 16 days, while 100% of mice returned to normoglycemia after 19 and 21 days respectively. These data show that local overexpression of VEGF in beta-cells improves islet engraftment and suggest that this is probably due to better islet cell revascularization.

7. From the in vitro experiments with the new genetically engineered beta cells we can conclude that the CDM3D-TET-VEGF cells show stable VEGF-A secretion which can be successfully controlled with tetracycline.

8-9. The regulated delivery of VEGF-A from CDM3D-TET-VEGF cells resulted in well measurable in vitro angiogenesis in our three-dimensional model of co-culturing beta cells with bovine endothelial cells. This process was well controlled with tetracycline. Switching-off VEGF gene expression using the TC system resulted in a near complete inhibition of the VEGF-induced angiogenic process. So, we have shown that potent and regulated delivery of VEGF can be achieved, and that we can increase the capacity of these cells to induce neovascularization in vitro.

10. The genetic modification did not affect the in vitro glucose stimulated insulin secretion of the newly created CDM3D-TET-VEGF beta-cell clusters when compared with control CDM3D cells.
11. To assess the role of VEGF overexpression on glycemic control by transplanted beta cell clusters, restoration of normoglycemia and dynamic changes in blood glucose concentrations were evaluated. The time required for diabetic mice to return to normoglycemia and stimulated plasma glucose clearance were also significantly accelerated in mice transplanted with CDM3D-TET-VEGF cells when compared to control cells. When clusters of CDM3D cells (control group) were transplanted, 50% of mice returned to normoglycemia after 30 days and 100% after 38 days. When clusters of CDM3D-TET-VEGF cells were used, the time to achieve normoglycemia was reduced: 50% of mice returned to normoglycemia after 20 days, while 100% of mice returned to normoglycemia after 24 days, respectively. The difference was statistically significant (p<0.05). The clearance of plasma glucose during IPGTT was accelerated in TET-VEGF group vs. the CDM3D-group (p<0.01).

12. With regard to intra-graft vascular density, the density of BS-1-stained cells was significantly greater in grafts of CDM3D-TET-VEGF cells when compared to CDM3D grafts (p<0.005). So, the increased expression of VEGF resulted in a significant increase in vascular density in grafted CDM3D-TET-VEGF-cells vs. control cells, and this was associated with an increase in graft cellular mass.

13. The secretion of VEGF and consequently the islet cell revascularisation could be controlled by adding Tetracycline to the recipients. VEGF was only needed during the first 2-3 weeks after transplantation; when removed, normoglycemia and graft vascularization were maintained. Tetracycline-treated mice transplanted with Tetracycline-treated cells failed to restore normoglycemia and to correct plasma glucose levels on IPGTT. This approach allowed us to maintain the VEGF overexpression until the revascularisation of the graft was completed and to switch-off the VEGF secretion with Tetracycline when the desired effects had been achieved.
5. CONCLUSIONS, SUMMARY OF NEW RESULTS

According to the results of the enzyme studies it can be stated that the data we gained provide new information for the introduction of a new enzyme to be used in human islet cell isolation. Digestive activity of the combination of Collagenase NB1 and Neutral Protease NB1 proved to be more calculable than that of the Liberase H1 product, used in the general practice. Separation of collagenase and neutral protease presented an additional benefit since the proportion of components was changeable. This way, composition of the enzyme product can be adjusted to the given pancreatic tissue. If the pancreas is extracted from a young donor, containing little connective tissue, a small amount of neutral protease can be given, while in the case of a more compact, fibrotic pancreas an increased amount can be applied. This can be important even in the cases of islet cell autotransplantation, since the removed tissue affected by chronic pancreatitis mostly contains an increased amount of connective tissue. Separation of the two components can extend the storage life of the product, as the activity of combined enzymes may fall when stored.

The most important feature and practical benefit of the new enzyme is that it enriches the opportunities of human isolation and makes the execution of "individual" islet cells isolation, adjusted to the characteristics of a given pancreas possible. This way, the efficacy of human islet isolations can be improved, and the number of donors suitable for islet cell transplantation can be increased.

The most significant finding from the VEGF overexpression studies is that our approach enabled drug-controlled, reversible VEGF expression after CDM3D cell cluster transplantation. VEGF secretion was only required during the early post-transplantation period; when subsequently inhibited by the addition of Tetracycline, normoglycemia and graft vascularization were maintained. Tetracycline-treated mice grafted with Tetracycline-treated cells failed to restore normoglycemia. Our new system allowed us to turn off VEGF expression once the desired effect had been achieved. Moreover, this presents a novel strategy to promote early revascularization and could be an attractive means of improving engraftment after islet cell transplantation.

Angiogenesis stimulated with VEGF gene therapy has been successfully applied in clinical studies relating to coronary and peripheral vessel diseases. The pharmacologically controllable VEGF-expression model developed by us could contribute to a reduced islet cell
loss following the transplantation, and given the rapid development of gene therapy it could serve as a base for the elaboration of a similar clinical method in islet cell transplantation as well.
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