Pancreatic islet transplantation: studies on the technique and efficacy of islet isolation and transplantation

Burg, M.P.M. van der

9 Summary and future prospects

Introduction (Chapter 1)

Pancreatic islet transplantation aims to reinstate normal blood glucose regulation in patients with insulin-dependent diabetes mellitus (IDDM) to improve the quality of life, and prevent, postpone or ameliorate the long-term crippling complications of the disease and, thereby, promote longevity of the patients. IDDM, also called type-1 diabetes, affects 0.2–0.4% of the population in industrialised countries. In these patients a chronic high blood sugar level (i.e. diabetes) is the consequence of destruction of the ß cells in the islets of Langerhans, which normally produce the insulin that is required to regulate the blood sugar level. The normal human pancreas contains roughly 1 million of these islets — which are tiny clumps of cells, up to 0.5 millimetre in diameter — scattered throughout the organ. Together the islets weigh approximately 1 gramme.

Conventional treatment with daily insulin injections in IDDM patients sustains the patient's life, but does usually not prevent a chronic high blood glucose level which in roughly 50% of the patients after 20-30 years may cause serious damage to the eyes, kidneys, nerves, and blood vessels — which threatens vision, eventually leads to kidney failure requiring dialysis or a renal transplant, and may lead to amputation of the lower limbs. The overall morbidity accounts for a one-third reduction of the overall life expectancy of the insulin-dependent diabetic patients. The importance of tight control of the blood sugar level to prevent or postpone the long-term diabetic complications has been conclusively shown in selected patients, but new strategies are required for broad implementation of tight control in the diabetic community. Considerable efforts are focused on both the education of patients for self-control of the blood sugar level, and development of new insulin injection or infusion devices to normalize blood sugar levels. The current insulin therapies, however, do not mimic the moment-to-moment adjustion of the secretion of

insulin and other hormones by the normal islets.

At present pancreas transplantation is the only way to reach normal blood sugar control over prolonged periods of time in diabetic patients. Further, with a well functioning graft, the progression of the chronic complications is halted or reversed, and the patient's quality of life improves, primarily by eliminating the need for frequent insulin injections and blood glucose measurements, and the risks of serious hypoglycemic episodes — i.e., life-threatening low sugar levels, commonly experienced by diabetics. However, because transplantation of the pancreas requires major surgery and life-long immunosuppressive therapy, this operation is, in general, only considered for the minority of IDDM patients who need a kidney transplant for renal failure — and the concomitant immunosuppressive therapy to prevent rejection. Since actually the pancreatic islets produce the insulin, and since the islets can be isolated from the pancreas in the laboratory using digestive collagenase solutions, transplantation of only the islets potentially offers many advantages over pancreas transplantation. Firstly, implantation of the isolated islets is simple and safe, consisting of little more than an injection of a few millilitres of an islet suspension. Secondly, the islets can be stored frozen in 'banks' or cultured, allowing: (i) the collection of islets from multiple donors for a transplant, (ii) sophisticated matching of the donor and recipient to reduce the risk of immune rejection, and (iii) all kinds of manipulations in the laboratory prior to the transplant (e.g. irradiation, or encapsulation of islets in semipermeable membranes) which may lower or eliminate the need for immunosuppressive drugs to prevent rejection of the islets — either human islets or even islets from animals such as the pig, in order to overcome future donor shortage.

During the past few years over 100 transplants of human islets in diabetic patients with advanced complications were attempted. Roughly 10–20 % of these recipients of human islet grafts could stop the insulin injections temporary, and 3 patients were insulin independent for over 2.5 years. Thus, the feasibility of this approach has been established. As yet, however, many problems need to be solved and much work has to be done before wide-spread application of this technique in type-1 diabetic patients before the onset of the devastating complications will become available. The consistent isolation of adequate numbers of pure islets for transplantation is a major problem, and the crux of islet transplantation, i.e. the thesis that diabetic complications can be halted or prevented through the continuous precise glycemic control by grafted isolated islets — which entails that long-term high quality metabolic control is achievable after transplantation of isolated islets — has not been established. These latter two issues were addressed in the studies in this thesis.

Donor effects on islet isolation outcome (Chapter 2)

A major problem hampering large-scale human islet transplantation programmes is the variability of islet isolation outcome — necessitating a variable number of donors. Due to the intertwined effects of numerous variables such as predonation events, donor and pancreas characteristics, organ preservation conditions, and isolation methods, the variability of isolation outcome is difficult to analyse. Knowledge of the variability attributable to intrinsic factors such as the islet content of the individual pancreas, is an essential prerequisite for analysis of the relative importance of the many extrinsic variables, which can be controlled. As yet however, the impact of the islet content of the pancreas and other donor-related variables on isolation outcome have not been evaluated systematically so far — either in large animals, or in man. We therefore studied the impact of these variables on the outcome of collagenase isolation of islets from the splenic part of the pancreas of 31 beagle dogs. The islet volume of the splenic pancreas averaged 15.7 µl per gramme pancreas, and varied threefold (from 8.4 to 27.3 µl). Isolated islet yield averaged 7.6 µl/g and varied ninefold (1.8–16.3 µl). The animals also varied in age eightfold (8–67 months) and body weight twofold (8.6–18.3 kg). The combined effect of interindividual differences in body weight and age explained 60% of the variability of the fractional islet volume of the pancreas. The fractional islet volume increased with body weight, and decreased with age. The interindividual differences in pancreatic islet content as well as body weight and age explained approximately 50% of the variability of islet yield after collagenase digestion and dispersion. Age and body weight of the animal did not affect the efficacy of the collagenase isolation of islets in this study. We conclude that the outcome of islet isolation may be fairly predictable after controlling for the variable islet content of pancreases, and other donor-related variables such as body weight and age.

Assessment of islet isolation (Chapter 3)

The outcome of islet isolation is considered uncertain, on the one hand because of the numerous extrinsic variables that potentially affect the outcome, and on the other hand because of the large variability of outcome, per se — in terms of e.g. the insulin recovery, and the size and yield of isolated islets. In the past, the efficacy of isolation techniques has been estimated indirectly, by comparison of the insulin extracted from samples of the donor pancreas and isolated islet suspensions. However, because islet and insulin recovery are generally expected to be affected differently by the many variables during isolation,

insulin extraction has been largely superseded by the current method of sizing islets to assess the total volume of harvested islets. Morphometric assessment of islet yield has facilitated the comparison of results from different laboratories and definition of the critical islet mass for successful transplantation. As yet, no attempts have been made to extend the use of morphometry for assessment of isolation efficacy by comparison of the isolated islet population with the native islet population of the pancreas — and, consequently, no comparison of islet and insulin recovery has been made either.

We addressed the efficacy of isolation by both morphometry and insulin extraction — using a simple and gentle technique of islet isolation in 31 dogs, to exclude extrinsic factors as much as possible. Islet isolation was performed by intraductal stationary collagenase digestion of the splenic segment of the dog pancreas. Tissue was manually dispersed in cold University of Wisconsin solution (UWS) and the resulting digest was purified by density gradient centrifugation. Samples from the pancreas, the digest suspension, and the purified (islet) and non-purified (acinar) fractions of the gradients, were compared by morphometry of islet volume and the islet size distribution, by insulin and amylase extraction, and by microscopy for morphology and to assess ß-cell granulation as a parameter for the insulin content. Viability of isolated islets was assessed by glucose stimulation during in vitro perifusion.

In contrast to a ~90% recovery of pancreatic insulin and amylase after digestion, islet yield amounted to 50% of the islet content of the pancreas. After density separation, nearly all of the recovered islets were found in the purified fraction — which contained virtually no acinar tissue. In contrast, half of the recovered insulin was located in the non-purified (acinar) fraction of the gradients — indicating that a substantial proportion of islets remained entrapped in non-digested pancreatic fragments. Correlation analysis demonstrated that ~50% of the variance of both islet and insulin yield after collagenase digestion may be attributed to interindividual differences in the islet and insulin content of the splenic pancreas. The insulin content of digest suspensions did neither correlate with islet nor insulin recovery in the purified fraction of the gradients, as opposed to the islet content of digest suspensions, which correlated with both the islet and insulin recovery in the purified gradient fractions. Theoretically degranulation of ß-cells during islet isolation could also have contributed to the poor correlation of the insulin content of digest and purified islet suspensions. However, the ratio of insulin and islet volume measurements in the pancreas and during the isolation and purification procedure, demonstrated no loss of insulin from the islets; and no histological evidence for ß-cell degranulation was found, either. A high

proportion of small islets after isolation has generally been considered a sign for islet fragmentation. In our study, however, comparison of the size distributions of isolated and native islets demonstrated a similar high proportion of small islets and no signs of fragmentation as judged by the largest and average diameter of the islet population.

We conclude that the variability of islet yield and size may be attributed to a large extent to the variability of the native islet population. Isolation efficacy was best documented by morphometry, because insulin recovery did not discriminate between isolated islets and islets entrapped in acinar tissue. Assessment by both morphometry and insulin extraction, however, demonstrated that we subjectively had underestimated the proportion of entrapped islets during islet sizing and further documented islet integrity by demonstrating preservation of the insulin content of isolated islets. We suggest that similar studies should be done in man to facilitate the analysis of other factors affecting the outcome of islet isolation for transplantation.

Cell preservation in UWS during islet isolation (Chapter 4)

After collagenase digestion of the pancreas, islet isolation is traditionally performed in physiological salt based solutions such as Hanks' balanced salt solution (HBSS) and RPMI culture medium, under hypothermic conditions — to inhibit the action of collagenase and slow down degenerative processes. Potentially detrimental effects of hypothermia when the cell is exposed to a physiological milieu — such as cell swelling, and eventually loss of integrity of the cell membrane and cell death — are well documented in studies designed for the hypothermic preservation of whole organs. The University of Wisconsin solution (UWS) has been primarily designed to minimise the side-effects of hypothermia during cold storage of the pancreas.

We hypothesised that the UWS would be also more appropriate for islet isolation and compared the outcome of islet isolation at 4°C in either the UWS or RPMI, and the final outcome after subsequent density gradient separation. Samples taken from the splenic pancreas, the digest, and from the gradient layers, were compared by morphometry and amylase extraction to assess the recovery of islet and acinar tissue, and purity. The isolation solution did not affect islet yield before purification which averaged 51% of the native islet mass. Loss of amylase (30%) and swelling of the acinar cells were observed in RPMI. In contrast, no loss of amylase and slight shrinkage of the acinar cells were observed in the UWS. The changes in cell volume were reflected by the apparent density and viability of islets and acini in the density gradients.

First, conventional hyperosmotic density gradients of dextran in HBSS were used for islet purification — because hyperosmolality of the density solution has been shown essential for purification. The densities of islet and acinar tissue were higher in the dextran gradient after isolation in the UWS compared to RPMI. However, near complete absence of acinar tissue in the major islet fractions of the gradient after isolation in UWS, and overlapping densities of islets and acini after isolation in RPMI, demonstrated the acinar cell to be more susceptible to cell swelling in the physiological solution. Dextran density separation resulted in a 15% purity and 41% recovery of the islets that had been isolated in RPMI, as compared to a 93% purity and 52% recovery of islets isolated in UWS.

Cell density and the outcome of density separation will be affected also by the osmolality of the density solution. Thus — to clearly delineate the impact of the UWS on the regulation of cell volume and the apparent density of islet and acinar tissue — the hyperosmotic dextran gradient was replaced by a novel normosmotic density gradient of Percoll in UWS. Percoll density separation improved the purity (99%) and recovery (74%) of islets isolated in UWS. These findings demonstrated the impermeant nature of the components of the UWS to be both necessary and sufficient to prevent cell swelling and obtain complete purity in the absence of hyperosmolality. Islets isolated in UWS further demonstrated a superior basal and glucose-stimulated insulin release during perifusion. Electron microscopy demonstrated a well-preserved islet ultrastructure after isolation in both solutions — except for slightly swollen mitochondria after isolation in RPMI. Autotransplantation of islets in pancreatectomised dogs was successful both after isolation in UWS and RPMI.

We conclude that the density and viability of islet and acinar tissue were best preserved by substitution of the UWS for conventional solutions during hypothermic isolation and purification. Prevention of cell swelling during hypothermic isolation in the UWS and consequently conservation of both the viability of islet and acinar tissue, as well as the density difference between islet and acinar tissue, would account for the improved recovery of virtually pure islets after density separation. Thus, prevention of cell swelling during islet isolation in UWS should facilitate (i) the analysis of other variables — such as predonation events and organ preservation conditions — that may affect the outcome of islet isolation, and (ii) the adjustment of osmolality and viscosity of density gradients to obtain similar results in man.

Duct-obliterated pancreatic islet transplantation (Chapter 5)

Duct-obliterated segmental pancreas transplantation is a widely applied islet replacement technique in diabetic patients. The safety of this method is the most important advantage, but the long-term glucose control is controversial. Both duct occlusion induced fibrosis and pancreatic mass reduction have been emphasised to be the major determinants in the deterioration of glucose regulation in previous experiments. In the study presented in Chapter 5 the separate and additive effects of these aspects of segmental ductobliterated pancreas transplantation were addressed.

Fasting, postprandial and intravenous glucose-stimulated glucose, insulin, glucagon, pancreatic polypeptide (PP), cholecystokinin (CCK) and intravenous bombesin-stimulated PP were studied in beagle dogs at three successive intervals in a crossover design: first at 6 wk following ~70% pancreatectomy with regular enteric exocrine drainage from the duodenal pancreatic remnant, next at 2 wk after venous transposition with systemic delivery of pancreatic hormones, and finally at 6 wk following in situ duct obliteration of the remnant. Following partial pancreatectomy we observed a 50% reduction in glucose tolerance and the second-phase insulin response to intravenous glucose stimulation. Venous transposition is preferred for technical reasons in pancreas transplantation. The peripheral insulin levels doubled upon venous transposition with systemic delivery of pancreatic hormones, which is undoubtedly the result of bypassing of first-pass hepatic insulin extraction. Other parameters were not dramatically affected after these operations. The most dramatic changes in glucose regulation were observed after in situ duct obliteration. At intravenous glucose stimulation a 50% reduced glucose tolerance was associated with a 50%-70% reduction in the overall and acute insulin responses, respectively. Duct obliteration induced sustained, fasting and postprandial (~8 mM) hyperglycemia. Fasting hormones and — of note — postprandial insulin, glucagon and CCK were not affected. The postprandial PP response was severely reduced and the bombesin-stimulated PP release was abolished by duct obliteration.

Although there are convincing data to show that duct obliteration interferes with endocrine function, it has not been clarified as yet how glucose regulation is affected. Postprandial 'normoinsulinemia' with ductobliterated remnants as opposed to a mean 50% reduction of the intravenous glucose-stimulated insulin response may be explained both from the different stimuli with these tests, as well as from the postprandial contribution of the enteroinsular axis. Since the postprandial CCK response was not affected by duct obliteration with exocrine

substitution and since CCK is known to have a progressively greater stimulatory effect on insulin release with higher glucose levels, normo- to hyperinsulinemia might be attained only as a consequence of a postprandial hyperglycemia enhanced incretin effect. Since quantitatively peripheral postprandial insulin was not affected by duct obliteration though insulin delivery was insufficient with respect to the prevailing glycemia, and since intravenous glucose-stimulated acute release was affected, fine regulation of insulin release seems deranged. Previous studies demonstrated that duct obliteration apart from inducing atrophy of exocrine tissue, disrupts islet architecture. The present finding that the PP response to bombesin stimulation was abolished by duct obliteration is suggestive for intrinsic denervation of islets as an important effect of duct obliteration. This suggestion is supported by a remarkable fall of the first-phase insulin response. Since duct obliteration mimics the effects of denervation on ß-cell and PP cell function, and interferes with normal islet morphology we suspect obliteration-induced intrinsic denervation of ß-cells to interfere with normal pulsatile insulin delivery, explaining for postprandial hyperglycemia with normo- or hyperinsulinemia. Although pancreas transplantation necessitates extrinsic denervation, intrinsic innervation and intrinsic fine regulation of insulin release might survive in the non-obliterated graft. Whether such a mechanism exists requires further investigation.

In vitro effects of gut hormones on isolated islets (Chapter 6)

The postprandial release and action of gut hormones, called "incretins", is held responsible for the alimentary augmentation of the insulin response to glucose. Our metabolic studies in dogs suggest that the incretin effect — which, normally accounts for roughly half of postprandial insulin — may account for most (~80–90%) of the insulin released during mild postprandial hyperglycemia after pancreatic islet transplantation. Insulinotropic effects of physiological levels of the gut hormones cholecystokinin (CCK), gastric inhibitory polypeptide (GIP), and most recently glucagon-like peptide-1 (GLP-1) have been documented in vivo and in the isolated perfused pancreas, in various species — but effects of physiological levels of these peptides on isolated islets are not well established. We, therefore, investigated whether physiological levels of these hormones, stimulate insulin release during perifusion of short-term (2-day) cultured canine isolated islets at glucose levels as observed postprandially after islet transplantation. Dose-response studies with CCK and GIP during perifusion at a 7.5 mM glucose level demonstrated a

transient insulin response to pharmacological levels of CCK, and a dose-dependent insulin output during physiological to pharmacological GIP stimulation. Perifusion with physiological levels of CCK, GIP, and GLP-1 at 2.5, 7.5, and 10 mM glucose levels demonstrated no effects of CCK, an 1.1 and 1.2-fold GIP-enhanced insulin release at 7.5 and 10 mM glucose respectively, and a maximum effect of GLP-1 from 7.5 mM glucose, resulting in a twofold insulin output.

Pharmacological, but not physiological, CCK levels stimulated insulin release in our model in concert with recent in vivo studies in dogs and man. Our finding that physiological levels of GIP and GLP-1 potentiate glucose-stimulated insulin secretion corroborates in vivo studies in man and studies of the perfused rat pancreas. GLP-1 was a considerably more potent incretin than GIP in our model. The finding that GLP-1, in contrast to GIP, is effective from a low glucose level corroborates recent reports that (near-)physiological doses of GLP-1, in contrast to GIP, are effective during basal glycemia in man. This, and our finding that the maximum effect of GLP-1 was also attained at a lower glucose level than observed with GIP, substantiate the contention that GLP-1 is a major incretin during euglycemia.

In contrast to these findings, pharmacological concentrations of GIP, and GLP-1 were required to stimulate insulin release from freshly isolated islets in previous studies. In our study, however, the islets were cultured two days before perifusion, which indicates short-term culture of islets of paramount importance in the response to physiological levels of these peptides. In conclusion isolated islets do respond to physiological ß-cell stimulation with gut peptides. Our findings indicate that CCK is not an incretin in the dog, and corroborate the notion that GLP-1 is a major incretin during euglycemia. The physiological role of GIP during euglycemia is equivocal. However, both GIP and GLP-1 may contribute to a hyperglycemia-enhanced activation of the enteroinsular axis after pancreatic islet transplantation, and other situations (e.g. type-2 diabetes) characterised by elevated glucose levels.

Function and survival of transplanted islets (Chapter 7)

Physiological glucose regulation is of utmost importance for prevention or halting the progression of secondary diabetic complications, but as yet metabolic control by islet grafts has not been well established. We, therefore, studied metabolic control after fasting, intravenous glucose, mixed meals, and an intravenous arginine bolus during 35 mM glycemia, before operation and up to three years after intrasplenic autotransplantation of isolated islets in six dogs.

In addition, the pancreatic polypeptide (PP) response to intravenous insulin-induced hypoglycemia was measured — a vagal, cholinergic, mediated stimulus — to monitor possible cholinergic re-innervation of the graft.

The islet dose at transplantation ranged from 1588–9065 islets per kg body weight (standardised islets, size 150 μm). A recipient of only 1588 highly purified islets per kg, became overt fasting hyperglycemic within a few days. All other animals received > 3000 islets per kg (i.e. >20% of native islet volume) by varying the purity of the grafts from ~5 to 100% which led to packed graft volumes of 14 to <0.3 ml. Four recipients of grafts with a volume ≤ 6 ml became fasting normoglycemic within 2 weeks. Time to normoglycemia was delayed to five weeks in the recipient of a 14 ml graft. Microscopic examination of the spleens demonstrated a normal histology in recipients of the ~100% pure grafts, but prominent scarring, and fibrotic reaction surrounding islets after failure of the partially purified grafts. The individual postprandial insulinogenic index (ratio of 2-h postprandial insulin to glucose levels) at one month posttransplant predicted (r = 0.99) the time to functional graft failure (6–175 weeks). Metabolic studies at six months in four dogs demonstrated normal fasting glucose and hormone levels, except for reduced PP levels. Intravenous glucose- and arginine-stimulated insulin were reduced to 15 % of preoperative values. By contrast, a normal postprandial insulin response was observed, albeit at moderate hyperglycemia (~10 mM). Postprandial glucagon and GIP had increased. Comparison of the posttransplant insulin responses to a meal and intravenous arginine demonstrated similar — close to maximum — stimulation of the graft by a meal. No PP response to intravenous insulin induced hypoglycemia was observed — indicating persistent absence of cholinergic re-innervation.

In the autotransplant model important factors to be considered for functional graft survival are both the dose, purity, as well as the metabolic state of the recipient. The prolonged fasting normoglycemia after transplantation of >3000 IEq/kg body weight corroborated reports of the threshold islet dose for successful autotransplantation in dogs. The importance of purification of the islet graft is controversial. Our findings that a small graft volume was associated with longevity of the grafts, and that fibrosis was restricted to the partially purified grafts, support the contention that contaminating acinar tissue may impair islet engraftment. The acute insulin response to arginine at 35 mM glycemia is known to elicit a close to maximal insulin response. Considering that after islet transplantation (i) the postprandial insulin response was at least as challenging as the latter test, and (ii) the postprandial insulin level probably depends on both the insulin secreting capacity as well as the

prevailing glycemia, and finally (iii) overt hyperglycemia per se may precipitate early failure of grafts: it is not surprising that a close correlation of the 1 mo postprandial insulinogenic index and time to graft failure was observed.

The postprandial normo-insulinemia as opposed to the ~85% reduction of the intravenous glucose- and arginine-stimulated insulin response in the established autografts may be explained both from the different stimuli with these tests, as well as from the postprandial contribution of the enteroinsular axis. Our recent in vitro perifusion studies demonstrated that physiological, circulating, levels of the insulinotropic gut peptides GIP and GLP-1 potentiate glucose-stimulated insulin from isolated perifused canine islets. Thus, since the postprandial GIP response had rather increased after islet transplantation, the normo-insulinemia was probably attained as a consequence of a postprandial hyperglycemia enhanced activation of the enteroinsular axis.

Although postprandial hyperglycemia as outlined above may account for postprandial normo-insulinemia, these glucose excursions remain to be explained. Apart from the reduced beta cell mass, both a deranged insulin secretion and PP deficiency may have been conducive to glucose intolerance. Firstly, the cholinergic mediated preabsorptive insulin response — which is known to be important for postprandial glucose tolerance — is either absent or drastically curtailed due to persistent cholinergic denervation of the grafts — as indicated by the abolished PP response to hypoglycemia. Second, a deranged pulsatile insulin secretion from isolated islets has been reported, and small changes in the periodicity are known to affect insulin sensitivity, and glucose handling. Finally, apart from a deranged insulin secretion, PP deficiency may be related to impaired glucose handling after islet transplantation. Previous studies of exogenous PP administration in PP-deficient animal models suggest that the postprandial PP response may be important to mediate the inhibiting effect of insulin on hepatic glucose production.

In conclusion, both the islet dose, purity of the graft, and the resultant glycemia may determine graft life. Both the insulin secretory capacity of the grafts, graft's life expectancy, and glucose regulation were best documented by meal testing. Tentatively, a postprandial hyperglycemia-enhanced incretin effect of GIP and other gut hormones may account for the difference in the insulin response to intravenous glucose and a meal; and both a reduction of the insulin secreting capacity, a deranged pulsatile delivery of insulin, as well as PP deficiency may have contributed to the moderate postprandial hyperglycemia. The reasons for spontaneous failure of islet grafts within a few years in the present and other studies are not clear, but larger doses of purified islets are

expected to extend graft life.

Insulin secretory capacity, insulin action and the enteroinsular axis after islet transplantation (Chapter 8)

Our previous studies suggested that after isolated islet transplantation, both a reduced insulin secretory capacity due to a subnormal islet dose, and a deminished insulin action due to a deranged fine regulation of insulin secretion may lead to postprandial hyperglycemia. The issue of the relative contribution of the insulin secretory capacity and the action of insulin in the postprandial glucose regulation has not been settled, yet. Further, we hypothesized that the graft's normo-insulinemic response to a meal, as opposed to the hypo-insulinemic response to intravenous substrates, may largely be attributed to a hyperglycemic potentiation of the enteroinsular axis — rather than the postprandial hyperglycemia per se. Glucagon-like peptide-1 7–36 amide (GLP-1) is now considered the major insulinotropic gut factor in the enteroinsular axis, and both GLP-1 and other gut hormones such as gastric inhibitory polypeptide (GIP) are known to have a progressively greater potentiating effect on glucose-stimulated insulin release at increasing ambient glucose levels. We previously demonstrated that physiological levels of GIP and notably GLP-1 enhance the insulin secretion from freshly isolated (short-term cultured) canine islets during in vitro perifusion at glucose levels that mimic the postprandial glycemic range after islet transplantation. As yet, however, no studies of the in vivo insulinotropic effects of gut hormones on long-term transplanted islets have been published.

We, therefore, studied the insulin secretory capacity (ISC) by intravenous arginine stimulation during 35 mM glucose clamps, the action of insulin during euglycemic insulin-clamps, and physiological meal stimulation, in 8 dogs at 6 months after intrasplenic autotransplantation of purified islets, compared with 30 controls. The enteroinsular axis was examined in the graft recipients by near-physiological infusion of GLP-1 under hyperglycemic clamp conditions that mimic the postprandial 8–9 mM glycemia observed after islet transplantation. The islet dose at transplantation averaged ~25% of the normal native islet mass, and the purity was ~70%. After grafting the ISC averaged ~25% of the control values. The postprandial insulin response, in contrast, had increased to 140% after transplantation — albeit with a concomitant mean glucose excursion to ~8.5 mM. Insulin action declined by 45% posttransplant. In the grafted dogs a significant negative correlation was observed between the ISC and both the postprandial glucose increment ($r = 0.9$) and insulin action ($r = 0.7$). Infusion of

GLP-1 potentiated (175%) the insulin response to the 8.5 mM glucose clamp.

The intravenous arginine challenge under similar hyperglycemic conditions has been shown to evoke near-maximal stimulation of the ß-cells and this ISC test is known to be a sensitive indicator of the ß-cell mass reduction in other models. The on average similar reduction of the islet mass and insulin secreting capacity to ~25% of the normal values after islet transplantation indicated the ISC to be likewise a sensitive indicator of the islet mass in our model. The insulinemia during the ISC test and the meal test was similar in the grafted animals, hence the grafted ß-cells were near-maximal stimulated by a meal, too. This conclusion is further substantiated by the negative correlation after islet transplantation of the insulin secretory capacity versus the postprandial glycemic increment. These findings confirm and extend our previous reports of the remarkable difference between the insulin response to intravenous glucose and a meal — both after islet transplantation, and after the in vivo isolation of islets following obliteration of the duct of the pancreas in dogs.

The insulinemia nearly doubled during GLP-1 infusion in our grafted dogs, and other workers have shown the same dose of GLP-1 to be less insulinotropic under milder hyperglycemic conditions in normal dogs. Thus, we conclude that a hyperglycemia-enhanced insulinotropic effect of GLP-1 — and probably other insulinotropic hormones like GIP, as well — may largely account for the hyperinsulinemic response to a meal after islet transplantation.

Postprandial hyperglycemia, as outlined above, may account for the postprandial insulinemia — the reason why the insulin secretion was inadequate to induce normal glucose levels remains to be addressed. Clearly, a reduced insulin sensitivity contributed to the postprandial hyperglycemia. Further, correlation of the graft's insulin secretory capacity with both the postprandial glucose excursion and insulin sensitivity in the grafted animals, indicated the graft's ß-cell mass to be the main determinant of the glucose intolerance. These findings corroborate studies in partially pancreatectomized animals, and newly diagnosed patients with insulin-dependent diabetes mellitus, that suggest that insulin resistance accompanies a reduced insulin secretory capacity, and furthermore may increase as the residual insulin secretory capacity decreases. Aside from a reduced insulin secretory capacity, an altered pattern of insulin secretion due to the absence of neural fine regulation of insulin release from the isolated islets may also have led to the impaired glucose tolerance and insulin resistance in the autografted dogs. Firstly, the largely or exclusively neurally mediated preabsorptive insulin response is important for postprandial glucose tolerance, and is probably absent or diminished after transplantation of isolated islets. Secondly, isolated

islets have been shown to secrete insulin in a deranged pulsatile fashion — which is known to lead to insulin resistance.

We conclude that both a diminished ß-cell mass and a qualitatively defective insulin secretion may contribute to insulin resistance and mild postprandial hyperglycemia after islet grafting. The diminished ß-cell mass appeared to be the main determinant of these defects. The insulinotropic effects of GLP-1 and probably other gut hormones, as well, may largely account for the marked difference in the insulin response to the intravenous and oral challenges. Thus, after transplantation of a suboptimal islet mass, postprandial glucose excursions are probably largely restrained by hyperglycemic potentiation of the enteroinsular axis, which can lead to close to maximum stimulation of insulin secretion — and, eventually, may lead to functional failure of the graft. Transplantation of a larger islet mass should allow prolonged near-normal glycemic control.

Conclusions and future prospects

Considerable effort has been devoted during the past few years to realize clinical islet transplantation. As noted in the introduction the feasibility of islet transplantation in humans has been demonstrated, but the obstacles that prevent its large-scale clinical application since the first rodent studies two decades ago are still the same: the islet isolation technique and rejection [1]. Isolation methods need to be developed further in order to consistently yield an adequate number of highly purified islets for transplantation. Further, although precise glycemic control is central to the concept of islet transplantation, as yet, the quality of metabolic control after islet transplantation has received comparatively little attention [2, 3]. The studies in this thesis addressed both technical aspects of the isolation procedure, and the metabolic control after islet transplantation in dogs. The autotransplantation model was chosen to circumvent the confounding side-effects of rejection and immuno-suppressive drugs [3–5].

The experiments that addressed technical aspects of islet isolation comprised a study of the causative factors of the extreme variability in islet isolation outcome in terms of yield, integrity, and purity of islets; and also introduced new methods to explain and reduce the variability of outcome — which hampers not only clinical application but also slows down technological innovation. In our study the major part of the variability of outcome could be attributed to the donor-related variability that exists prior to the isolation procedure. Donor-related factors such as body weight, age, and — of note — a

threefold variation of the islet content of the pancreas accounted for half of the variability of isolation outcome in our studies. Thus, assessment of the efficacy of islet isolation by morphometric assessment may accelerate, facilitate, and reduce the costs of the study of other factors that may also affect islet isolation outcome. Moreover, in contrast to the wide spread concern that islet fragmentation is an inevitable consequence of the isolation of islets in large mammals, the morphometric data demonstrated that islet integrity can be largely preserved using a gentle isolation method — at least in the dog. Additional assessment of insulin recovery as a simple alternative measure of the isolation efficacy, demonstrated the inadequacy of this biochemical method to discriminate between isolated islets cleanly cleaved from acinar tissue, and islets still embedded in pancreatic fragments. By using both assessment methods together, however, the latter valuable information was obtained, and further preservation of the insulin content of the purified islets was demonstrated.

More exciting was the finding — by serendipity — of the striking effects of the UWS as an isolation solution on tissue density and the subsequent density gradient purification of islets. The potentially adverse effects of cold preservation of tissue — although well-known in the organ preservation field — had not been recognised in the islet isolation field. Our subsequent studies of the beneficial effects of the UWS during islet isolation and density gradient purification, confirmed our hypothesis that consistent next to complete purity is obtained by prevention of swelling of islet and acinar cells — which is promoted during cold processing of the tissue in conventional physiological-salt based isolation solutions — and demonstrated further that a higher yield of viable islets is also obtained. Thus, in the experimental setting at least, the problem of purification of isolated islets has largely been solved. Admittedly, the additional factors related to organ procurement and storage, that may affect the outcome of human islet isolation, were circumvented in the dog work. However, preliminary human islet isolation data indicated similar beneficial effects of the UWS [6, 7], and the effectiveness of UW-isolation of human islets on the outcome of purification has been conclusively demonstrated recently by the Leicester group [8]. Now, most other groups have introduced the UWS for islet isolation from the pancreas of man and other species [8–18], or, alternatively, introduced the Euro-Collins organ preservation solution for islet isolation [19, 20], and purification [21]. The improved control over the conditions during islet isolation in the UWS should facilitate the identification and the control of other factors that may affect the islet isolation outcome. Currently the prevention or reversal of cell swelling during islet isolation and

purification appears to be the most important contribution of a preservation solution as islet isolation solution. We previously noted — to the best of our knowledge for the first time — that the convenient (commercially available) Ficoll-sodium-diatrizoate (FSD) density solution — which along with other radiopaque solutions has been widely favoured in many areas of cell separation because of the low viscosity and chemically inert components — may probably also contribute in islet purification by preservation of the volume of cells in the cold, because FSD — like the UWS — also contains a large molecular weight, impermeant, anion [7]. The latter preliminary report of simple highly efficient (~100% purity, and 80% recovery) neutral density purification of islets in FSD (1.119 g/ml) after islet isolation in the UWS, has been corroborated [22, 23] — and, the combination of islet isolation in an organ preservation solution and density purification in FSD or a similar solution will probably become the standard during the next years. Other important issues in the organ preservation field such as the buffering capacity of the preservation solution to prevent tissue acidosis [24] and the inclusion of free radical scavengers or cytoprotective substances such as polyethylene glycol [25, 26] might in the future prove equally important in islet isolation. A pressing need is the formulation of a solution that can be used during both the warm (physiological) and cold phases of organ procurement and cold storage, collagenase digestion at 37°C, and the isolation, purification, and rewarming steps — as well as, preferably, during a subsequent cryopreservation procedure. Currently different solutions during these different steps have to be used, and these solutions are difficult to change, and are inappropriate during the transition phases of warming and cooling. Cell and organ preservation studies with solutions that contain physiological concentrations of sodium and potassium as opposed to the inverse Na^+/K^+ ratio of the regular UWS [26–28], and the data with the physiological sodium based FSD solutions, indicate the concentration of these cations to be not important for cell volume preservation. Thus a physiological Na^+/K^+ ratio may be a first successful step in the design of a lactobionate based 'basic' UWS that may be used during both the warm and cold phases of islet isolation.

Our first indication that the anatomical isolation of islet cells from their natural environment may adversely affect glucose regulation was obtained in the study of effects of duct obliteration of the pancreas. The surviving islets after duct-obliteration-induced atrophy of the exocrine pancreas are not only — obviously — isolated from the exocrine tissue, but also become intrinsically denervated. The relative importance of the quantitative loss of insulin secreting capacity as a consequence of the loss of islets on the one hand and the adverse

qualitative effects as a result of the denervation and disrupture of the normal architecture of the islets following duct obliteration on the other, remains yet to be elucidated. In any case, the marked difference in the normal insulin response to meals and the severely reduced response to an intravenous glucose challenge indicated that insulinotropic gut hormones may play a more important role in the control of glucose regulation under the mild hyperglycemic conditions observed after pancreatic islet transplantation. The enteroinsular axis was therefore studied in more detail in the subsequent in vitro and in vivo studies of isolated islets.

Previously, direct insulinotropic effects on isolated islets of pharmacological rather than physiological concentrations of incretin candidates had been reported, therefore insulinotropic effects of GIP and particularly GLP-1 at physiological concentrations were established first in vitro during perifusion studies of isolated normal islets. In vivo studies with low-dose GLP-1 during hyperglycemic clamps that mimic the postprandial glycemic conditions as observed after islet transplantation confirmed the in vitro data and further demonstrated preservation of the insulinotropic effect of GLP-1 on long-term transplanted islets. These findings strongly suggested the hormonal branch of the enteroinsular axis to be the major physiological control mechanism for glucose regulation after islet transplantation.

Another interesting conclusion from the metabolic studies after islet transplantation was that the postprandial hyperglycemia leads to a chronic (close to) maximal pressure on the insulin secreting capacity of the grafts that comprised a clearly subnormal islet mass — which, eventually, may lead to functional failure of a graft. These findings suggest that exposure of intraportally transplanted islets — the preferred site in clinical practice — to the high intraportal levels of nutrients and gut hormones may be a contributing factor — along with the likewise high intraportal levels of immunosuppressive drugs and toxic substances — to the early failure of these grafts [3, 29–31]. Both an inadequate islet mass and the rather isolated state of the grafted islets, due to the persistent absence of innervation, contributed to a defective insulin secretion, glucose intolerance and the appearance of insulin resistance. Since the duct obliteration study a diminished action of insulin as a consequence of a deranged pulsatile insulin secretion due to the intrinsic denervation of islets was emphasised as a causative factor in the occurence of postprandial hyperglycemia. However, the present data indicate the islet dose to be the major determinant of both the glycemia and insulin action. Insulin resistance in the recipients, nevertheless, may precipitate the functional failure of the borderline-adequate islet grafts, which comprised — at the time of

transplantation — only ~25% of the normal native islet mass. These findings underscore the importance of striving for optimal metabolic control in type-1 diabetic recipients of islets grafts as advocated recently by the Giessen group [32] and suggested by the Islet Transplant Registry findings which indicate that underlying peripheral insulin resistance prevented the reversal of insulin dependence in many of the type-1 recipients of islet allografts — explaining for the presence of insulin-independence at one-year after transplantation in 23 % of the IDDM recipients as opposed to 43% of the recipients of islet grafts to prevent diabetes secondary to pancreatectomy at the time of transplantation [33].

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