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THE UNIVERSITY OF ALBERTA

COMPARISON OF TRANSPLANTATION SITES FOR FRESH AND CRYOPRESERVED CANINE PANCREATIC MICROFRAGMENTS

by

MARK G. EVANS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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OF MASTER OF SCIENCE

IN

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DEPARTMENT OF SURGERY

EDMONTON, ALBERTA

FALL 1988

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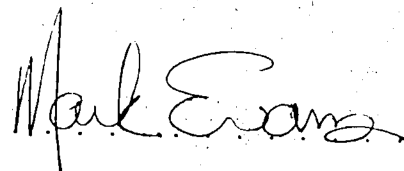

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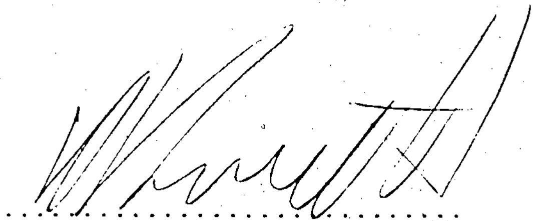

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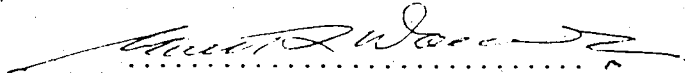

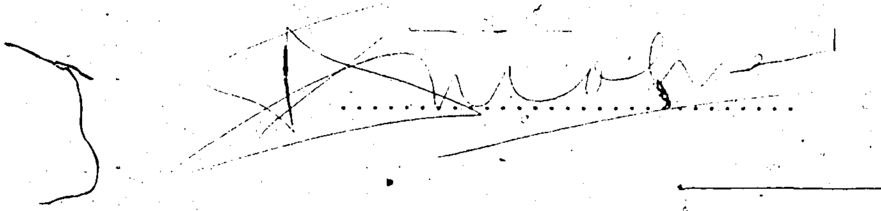

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This text is dedicated to

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ABSTRACT

Definition of the optimal recipient site for transplanted pancreatic islets and increasing graft purity are two issues to be clarified in order to bring successful clinical trials to fruition.

In the current study, surgical pancreatectomy was performed in dogs (n=40) following preoperative intravenous glucose tolerance test (ivGTT). Six dogs were maintained as apancreatic controls. Freshly isolated pancreatic microfragments were autografted by intrasplenic (n=10), intraportal (n=6) and renal capsular (n=6) injection and compared to dogs receiving intraportal (n=6) and renal capsular (n=6) grafts of cryopreserved tissue. Samples were taken from fresh pancreas, freshly isolated microfragments and cryopreserved microfragments for measurement of insulin and amylase content and the insulin/amylase ratio was used as an estimate of graft purity. Portal venous pressure was measured before and after intrasplenic and intraportal implantation. Function was assessed by fasting plasma glucose (PG) and insulin and by ivGTT 1 and 3 months following transplantation. Liver enzymes (Alk. Phos., SGOT, LDH), bilirubin and renal function (BUN, creatinine) were assessed perioperatively in all dogs and the coagulation system (PT, PTT, FDP platelet count) was monitored in dogs receiving intrasplenic and intraportal grafts.

Mean (±SEM) insulin concentration of fresh and cryopreserved grafts were 2083±200 mU/g and 2330±220 mU/g respectively. Amylase concentration of fresh graft was 2240±280 IU/g and 830±100 IU/g for cryopreserved tissue ($p<0.001$). Insulin/amylase ratios for fresh and cryopreserved tissue were 1.0±0.1 and 3.3±0.5 respectively ($p<0.001$). Portal pressure of dogs receiving fresh tissue rose 31.2±3.3 cm H_2O

compared to 16.4±2.3 cm H_2O in those receiving cryopreserved tissue ($p<0.01$) while intrasplenic implantation of fresh tissue produced no change. Long term normoglycemia occurred in: 9 of 10 dogs receiving fresh intrasplenic implants, 2 of 6 receiving fresh intraportal implants and 3 of 6 dogs receiving cryopreserved intraportal grafts. None of the dogs receiving renal capsular grafts became normoglycemic. The K values fell from 3.5±0.39 pre-operatively to 1.3±0.2, 1.4±0.4 and 1.0±0.2 in recipients of intrasplenic ($p<0.01$), fresh intraportal ($p<0.01$) and cryopreserved intraportal ($p<0.01$) grafts respectively after 1 month. Liver enzymes rose 24 h following engraftment and returned to preoperative levels by 1 month in all groups without effecting bilirubin. Renal function was not altered in any group and coagulation abnormalities were not seen following intrasplenic or intraportal implantation.

Purification of pancreatic microfragments is demonstrated by a greater than 3-fold rise in the insulin/amylase ratio following cryopreservation resulting in reduced portal hypertension following intraportal embolization. Intrasplenic implantation of pancreatic microfragments in a reliable technique for normalizing PG in pancreatectomized dogs while the intraportal route is associated with significant morbidity. The renal capsule is an unsuitable recipient site for canine pancreatic microfragments.

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ABBREVIATIONS

Alk. Phos.	Alkaline phosphatase
BUN	Blood urea nitrogen
CCK	Cholecystokinin
DIC	Disseminated intravascular coagulation
DMSO	Dimethyl sulfoxide
EGTA	Ethylene glycol tetra acetic acid
FDP	Fibrin degradation products
GIP	Gastric inhibitory peptide
HLA	Human leukocyte antigen
IDDM	Insulin dependant diabetes mellitus
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IRI	Immunoreactive insulin
ivGTT	Intravenous glucose tolerance test
KIU	Kallikrein inhibitory units
LDH	Lactate dehydrogenase
MHC	Major histocompatibility complex
NIDDM	Non insulin dependant diabetes mellitus
PG	Plasma glucose
PT	Prothrombin time
PTT	Partial thromboplastin time
SGOT	Serum glutamate oxalacetic transaminase
USP	United States Pharmacopeia

I

INTRODUCTION

Diabetes mellitus refers to a syndrome that is characterized by a broad array of physiologic and anatomic abnormalities most notably disturbed glucose metabolism resulting in inappropriate hyperglycemia (1).

The true frequency in the general population is difficult to ascertain because of differing standards of diagnoses but is probably approximately 1% (2). Of the 220 million people in the United States approximately 1.5 million, including 100,000 children are insulin dependent diabetics. An almost equal number take oral hypoglycemics and another 2 to 3 million control their disease by dietary measures alone (1).

The disease is characterized by a series of hormone-induced metabolic abnormalities: by long-term complications involving the eyes, kidneys, nerves, and blood vessels; and by a lesion of the basement membranes demonstrable by electron microscopy. In recent years it has become clear that a variety of syndromes are subsumed under the general term "diabetes" and that they differ in clinical manifestations and in patterns of inheritance.

Diabetes can be primary or secondary. Secondary causes include chronic pancreatitis, hormone abnormalities such as those which accompany pheochromocytoma, or Cushings disease, drugs and certain genetic syndromes. The classification of primary diabetes follows the recommendations of the National Diabetes Data Group (3). Patients who depend on insulin for the prevention of ketoacidosis have insulin

dependent diabetes mellitus (IDDM). This form is also referred to as Type 1 diabetes and often develops in childhood or adolescence. Non-insulin dependent (NIDDM) or Type II diabetics do not rely on insulin to ward off ketoacidosis. This form of the disease tends to occur in middle or later life, occurs predominately in obese individuals and comprises 90-95% of all diabetics.

It has long been accepted that there is a genetic component involved in the development of diabetes. However, the manner in which this is manifest is still a subject of great debate (1,2,4). It is probable that diabetes is genetically heterogeneous and that similar diabetic phenotypes may result from differing genotypes. Genetic factors play a role in all primary forms of diabetes mellitus, but their importance varies (2). In insulin-dependent disease the genetic factor(s) may be largely permissive while in non-insulin dependent diabetes they may be more nearly causal. This conclusion is based on extensive studies in monozygotic twins, which showed a 93% concordance rate for adult onset disease and a 50% concordance rate for juvenile onset disease (4). Therefore genetic factors appear to be predominant in NIDDM, but presumably environmental factors are needed to trigger the onset of IDDM.

The predisposing gene(s) in IDDM likely resides in the sixth chromosome in view of the strong association between diabetes and certain HLA antigens, specifically HLA-B8, HLA-B15, HLA-D3, HLA-D4 (1). The location of the predisposing gene(s) for NIDDM is not known, but speculation has focused on the eleventh chromosome which bears, on its short arm, the structural gene for insulin. It has been observed that some people have an insertion of extra DNA located near the insulin

structural gene and that its insertion occurs more frequently in NIDDM than normal controls or patients with IDDM (2).

As previously stated, studies in identical twins suggest the necessity for an interaction between genetic and environmental factors for the development of IDDM. Viruses have been primarily implicated (1,2,4). An association between viral infections and IDDM was originally suggested by seasonal variations in the onset of the disease and temporal association between the appearance of diabetes and preceding episodes of mumps, hepatitis, infectious mononucleosis and Coxsackie infections. The most direct evidence for a role of viral infection in the pathogenesis of IDDM relates to the recovery of Coxsackie virus from the pancreatic tissue of a child who died with meningoencephalitis and recent onset diabetes. The isolated virus caused beta cell destruction and diabetes when injected into mice (5).

The immune system is also implicated in the pathogenesis of IDDM. Autopsies of insulin dependent diabetics have demonstrated round cell invasion surrounding beta cells and the presence of circulating antibodies directed against beta cells, in the serum of newly diagnosed insulin dependent diabetics suggest an autoimmune role.

The pathogenesis of NIDDM is more complicated and speculative. Identical twin studies suggest a strong genetic component. Pathologic studies reveal a deficiency of beta cells, but functional aspects are also involved including a reduced rate of insulin release and a peripheral resistance to existing insulin, which may be genetically linked and unmasked by aging (4).

Complications of Diabetes

The complications of diabetes can be divided into two major categories, acute and chronic. The acute complications of uncontrolled diabetes are ketoacidosis and hyperosmolar coma, as well as an array of physiologic abnormalities including electrolyte abnormalities, increased red cell membrane stiffness, impaired phagocytosis by leucocytes, increased platelet aggregation, increased glomerular permeability and filtration, increased retinal microvascular permeability, lowered levels of high density lipoproteins, impaired wound healing, and decreased nerve conduction velocity (1,2,6). These acute complications can be attributed to relative or complete lack of insulin or its effectiveness and can be reversed by correcting the hyperglycemia with insulin (1).

Unfortunately, the diabetic patient is also subject to a number of chronic complications which cause significant morbidity and premature mortality, and occur despite of the use of exogenous insulin (7). The chronic syndromes can be divided into three groups: abnormalities involving large vessels (macroangiopathy or atherosclerosis); disorders which are unique to diabetics and involve arterioles and capillaries (microangiopathy) and a collection of abnormalities, many being unique to diabetics, involving the nervous system, skin and other parts of the body (1). Due to these chronic syndromes the life expectancy of a diabetic is two-thirds that of the general population. Atherosclerosis appears at an early age and is severe. Strokes are twice as frequent, myocardial infarctions two to ten times more frequent and peripheral vascular disease 50-100 times more frequent in diabetics.

The microangiopathy is seen primarily in the eyes and kidneys but has been noted elsewhere and occurs most commonly in IDDM. The diabetic

is 25 times more prone to blindness and partial loss of vision than the non-diabetic. Cataracts occur at 4 to 6 times the rate in diabetics and occur at an earlier age. One in 20 of all IDDM diabetics becomes blind (1).

Retinopathic changes are divided into two large categories: simple and proliferative. The earliest changes are that of increased capillary permeability. Microaneurysms follow with subsequent development of hemorrhage and exudate formation. Ultimately proliferative changes occur including neovascularization and scarring. Approximately 85% of diabetics eventually develop retinopathy to some degree (2).

The most serious life threatening complication of diabetes involves the kidney. Approximately one half of insulin dependent diabetics develop renal failure (1). The typical renal abnormality is a diffuse or nodular glomerulosclerosis (Kimmelsteil-Wilson lesion) which is characterized by a thickening of basement membrane in the glomerular capillary loops as well as accumulation of glycoprotein in the mesangial region of the glomerulus (6). Renal failure usually occurs 20-30 years after the onset of diabetes.

The neuropathic changes associated with diabetes are extremely variable. Every diabetic has demonstrable neuropathic sequelae after several years. About one in ten develop significant symptoms and one half of these have problems severe enough to be disabling. Problems include sensory discomfort and various autonomic abnormalities including, impaired bladder emptying, diarrhea, incontinence and impotence (8).

The exact cause of the chronic diabetic syndromes is unknown. It may be that hyperglycemia or an associated metabolic disorder causes or

accelerates the development of chronic complications or alternatively the complications may be primarily determined by genetic factors independent of hyperglycemia (2). The most suggestive evidence that the metabolic environment per se causes complications, in man, comes from the observation that kidneys from donors who have neither diabetes nor a family history of diabetes develop characteristic lesions of diabetic nephropathy within 3-5 years after transplantation into a diabetic recipient (2).

The problem of the relationship between the control of the metabolic deficit and the development of a long term sequelae is controversial and subject to debate (1,2,6,9). A prospective trial comparing absolute diabetic control to poor diabetic control and the development of chronic complications is not possible. As Tchobroutsky states, "the definitive demonstration that the microvascular complications of diabetes can be prevented by normalization of blood glucose in man cannot be made since normoglycemia cannot be sustained from the beginning of the disease with our current methods of treatment" (9). The literature with respect to man, therefor consists primarily of retrospective and prospective studies comparing the degree of diabetic control and duration of disease to the development of chronic complications.

Pirart studied over 4000 diabetic patients between 1947 and 1973 and concluded that retinopathy, nephropathy and neuropathy are true complications of diabetes, with chronic hyperglycemia as a common etiological factor (10). The Steno Study Group examined the effect of strict metabolic control of diabetes by subcutaneous infusion pump and demonstrated improved retinal and renal function over a six month period

(11). Eschwege et al. demonstrated a slower rate of progression of microaneurysms in insulin dependent diabetics t ced with multiple daily insulin injections compared to patients treated with a single daily injection (12). Miki and co-workers demonstrated a relationship between good control of diabetes and the slower progression of existing retinopathy (13).

There is an ample supply of experimental studies involving lower animals that support the hypothesis that the metabolic environment of the diabetic causes the complications (14,15,16). In dogs made alloxan-diabetic, Engerman et al., showed that microvascular retinal lesions were significantly reduced in frequency and severity in well controlled dogs compared to dogs deliberately poorly controlled (14). Lee and associates demonstrated that diabetic glomerular changes develop in normal kidneys transplanted into diabetic rats. Conversely, these changes are reversible upon transplantation of kidneys from diabetic rats into normal recipients (15).

On balance it should be concluded that while a causal relationship between hyperglycemia and the development of complications can be neither proved nor disproved, it is strongly suggested by the existing experimental data. As Foster suggests "it would appear prudent to maintain the plasma glucose as near normal as possible in all diabetic patients" (2).

The fact that many proteins can be glycosylated non-enzymatically and that the degree of glycosylation is directly correlated with the mean level of the plasma glucose provides a hypothetical mechanism by which hyperglycemia may cause chronic complications. It is presumed that the glycosylated peptides might cause abnormal structure and

function of vascular and other tissues (2). This type of evidence provides for the first time a biochemical rationale for meticulous control of glycemia.

Conventional Therapy

The first empirical treatment of diabetes mellitus is credited to John Rollo who, in 1797 prescribed a diet exclusively of meat and dietary manipulations continued to be the mainstay of treatment until 1921 (17). With the isolation of insulin, the modern era of treatment for diabetes began. Although certain strategies have evolved since that time dietary management and intermittent subcutaneous insulin injection are still the standard treatment of insulin dependent diabetes (1,2).

Ar of advancement in the delivery of exogenous insulin include home glucose monitoring, multiple daily injections of insulin, and the use of continuous subcutaneous insulin infusion pumps (18,19,20). With the use of home glucose monitoring many patients can be trained to become experts in maintaining near normoglycemia (18).

The failure of conventional treatment to normalize blood glucose levels in diabetics provided the impetus for development of a true mechanical endocrine pancreas. With such a system blood glucose levels are maintained within a target range by the infusion of insulin at a variable rate adjusted by on-line monitoring of blood glucose levels. Use of such monitoring systems has been limited because of technical problems in developing an implantable glucose sensor. Presently available systems utilize extracorporeal sensors that are bulky and require attachment of the patient to an intravenous line with continuous blood withdrawal. Due to these difficulties attention turned next to the use of insulin delivery systems that mimic normal insulin secretion

but that do not depend on minute to minute monitoring of blood glucose levels. With insulin infusion pumps insulin delivery is programmed to mimic the diurnal variation in plasma insulin and to provide pre-meal boluses. The subcutaneous route of insulin administration is preferable to the intravenous route because of greater safety and fewer complications (19). It has been shown in short-term in-patient studies, normal or near normal glucose control can be achieved with the use of such pumps. Subsequent studies have demonstrated the effectiveness of subcutaneous infusion systems in the out- patient management of diabetes (19). Although the control of glucose regulation attained by continuous infusion is approximately the same as intensive multiple daily injection therapy, patient preference and acceptability is higher with use of the pump (21).

Pump therapy has been associated with correction of a variety of metabolic, functional, and structural abnormalities that characterize poorly controlled diabetes (22). The ability of the pump system to stop or reverse the microvascular complications of diabetes has not been demonstrated (19). The insulin infusion system is susceptible to a variety of problems including catheter leakage or occlusion, dislodgement of the needle, battery failure and local infections. The most serious complication of the infusion system, or any method of maintaining close metabolic control with exogenous insulin, is hypoglycemia. Poorly controlled diabetics are vulnerable to hypoglycemia because they generally lack the glucagon response to a falling blood sugar which is the first line of defense against hypoglycemia in non-diabetic individuals (18).

The endocrine control of glucose metabolism is far more sophisticated than a reflex release of insulin in response to a rising plasma glucose. It is now recognized that in non-diabetic individuals, normoglycemia is largely maintained by the coordinated interplay of insulin and glucagon (18). The appropriate secretion of insulin limits the magnitude and duration of postprandial hyperglycemia and the secretion of glucagon prevents the occurrence of hypoglycemia between meals. The plasma glucose concentration and the overall movement of glucose to various tissues is dependent upon the relative amounts of insulin and glucagon at any given time. Therefore the proper coordination of insulin secretion to glucagon secretion is critically important. This is made possible by intercellular channels between adjacent alpha and beta cells known as gap junctions and by feedback relationships between each others hormones (18). When normally coordinated the alpha-beta unit provides a virtually fool-proof defense against both hyperglycemia and hypoglycemia.

In healthy fasting non-diabetic individuals insulin is secreted at a rate of probably less than 1 unit per hour to keep peripheral insulin levels in the range of 10-15 microunits per mL. Avoidance of hyperglycemia at mealtime requires a rapid and properly timed increase in the insulin secretory rate proportional to the size of the meal. To prevent postprandial hyperglycemia the burst of insulin must anticipate the influx of glucose from the intestine rather than simply react to a rising plasma glucose level. The glucose level itself is not normally the initiator of the insulin response early in the course of a meal. Insulin secretion begins before the first rise in arterial glucose levels (18). Clearly then, other signals, besides glucose must shape

both the timing and the degree of the insulin response to prevent excessive postprandial hyperglycemia. It would appear that several signals originate in the gastrointestinal tract, including vagal neurotransmission and a number of gastrointestinal "hormones" such as CCK, gastrin, secretin,and GIP. The importance of the hepatic portal circulation for insulin to maintain normal full metabolism has been demonstrated as well (23).

Given the complex nature of glucose homeostasis, which requires the intimate relationship between alpha and beta cells functioning as a single unit, it is not surprising that the administration of a single hormone, insulin, given in response to a single parameter, glucose, fails to provide optimal diabetic control. In spite of increasing sophistication in the fashion it is given, it seems unlikely that exogenous insulin administration will ever provide the strict control necessary to p event chronic diabetic syndromes.

Pancreatic Transplantation as Treatment

Total endocrine replacement therapy in the form of pancreatic transplantation was born out of the realization that exogenous insulin administration, as currently practiced, failed to control the development of long-term complications of diabetes.

Transplantation of the endocrine pancreas can be achieved in one of three ways, immediately vascularized whole organ transplants, segmental pancreatic. transplants or the transplantation of islets of Langerhans as a free graft (24). There is a wealth of experimental studies in small animals which demonstrate the ability of pancreatic grafts to prevent or reverse diabetic microangiopathy (16,24-32).

In 1974 Mauer et al. demonstrated for the first time the ability of islet transplantation to affect the progression of renal glomerular lesions (29,30). They described an increase in mesangial matrix of glomeruli and the deposition of macromolecules (IgG, IgM, complement) in the mesangium 6 months after the induction of diabetes. These investigators demonstrated that islets transplanted 6-9 months after the induction of diabetes caused a progressive disappearance of IgG, IgM and complement from the glomeruli. In addition, the amount of mesangial matrix either failed to increase or actually decreased after islet transplantation while in untreated rats the renal lesions progressed. Gray and co-workers (16), performed similar experiments and demonstrated that islet transplantation at the inception of diabetes would prevent the subsequent deposition of immunoglobulins in the glomeruli and thickening of the mesangium. Hoffman and co-workers compared fetal pancreas transplantation to insulin injection in rats and demonstrated that glomerular basement membrane thickening failed to develop in the transplanted rats while the insulin controlled rats developed glomerular lesions (26).

Leakage from retinal capillaries of diabetic patients can now be measured quantitatively after fluorescein angiography. Significant leakage has been demonstrated with this technique at the time of onset of diabetes in the juvenile diabetes. Krupin et al. (31), adapted this technique for the quantitative measurement of fluorescein leakage into the anterior chamber of the eye of the rat. In the diabetic animal, the amount of fluorescein leakage increased two fold compared to normal animals. Transplantation of islets into diabetic animals resulted in a reversal of this vascular abnormality and a return to normal within 10

days of transplantation. Gray (16), also demonstrated that the islet cell transplantation protected against retinal changes due to diabetes.

Nelson and associates have reported degenerative changes in autonomic terminals of Auerbachs and Meissners plexuses in the colons of diabetic rats (32). Transplantation of isolated islets one month after induction of diabetes completely prevented the development of these anatomic changes in the autonomic nervous system.

The findings that experimental islet transplantation will either prevent or reverse early diabetic complications involving the microvascular system of the eye, kidney and autonomic nervous system provides hope that the use of islet transplantation in diabetic patients may be helpful in arresting, preventing or possibly reversing diabetic complications in these individuals.

Pancreas Transplantation

The use of transplanted pancreatic tissue to ameliorate diabetes mellitus, is not a new concept. For almost a century, since the classic studies of von Mering and Minkowski (33) in 1889, which demonstrated that removal of the canine pancreas resulted in hyperglycemia, scientists have pursued pancreatic transplantation as a treatment for diabetes.

Hedon in 1892, described neovascularization of successful autografts of canine pancreatic segments placed subcutaneously on a temporary vascular pedicle (17). Normoglycemia was unaffected by division of the pedicle, and diabetes ensued following excision of the segmental pancreatic graft. On December 20, 1893, twenty-nine years before the pioneering work of Banting and Best, Drs. Watson Williams and Harsant performed the first human pancreatic transplant in Bristol (35).

They treated a 15 year old boy by the subcutaneous implantation of three pieces of freshly slaughtered sheep pancreas however the boy died of diabetic ketoacidosis three days later. Leonid Sobolev spent a lifetime of research dedicated to the study of the pancreas and is generally credited with being the first major proponent of transplantation of pancreatic tissue as a treatment of diabetes (36). Studies by Ivy and Farrell in 1926 and Houssay in 1929, documented short-term function and histological survival of canine pancreatic allografts (25).

The discovery of insulin by Banting and Best in 1922 and its purification by J. B. Collip shortly thereafter shifted the emphasis of research towards improving the administration of this new drug. However, with the realization that exogenous insulin does not control the microvascular complications of long-term diabetes, pancreatic transplantation has re-emerged both in the laboratory and also as a potentially therapeutic modality.

The research efforts and techniques employed to transplant pancreatic endocrine tissue follow two major paths, immediately vascularized grafts and free pancreatic islet transplantation. While technical barriers have prevented widespread and safe clinical application of both techniques the ultimate problem faced by each method is allograft rejection.

Whole Organ Transplantation

The first vascularized whole organ pancreatic transplant in dogs was performed by Lichtenstein and Barschak in 1957 (25). Increasing familiarity with pancreatic and vascular surgery led to a variety of

experimental studies in the canine model, followed soon thereafter by human trials.

The initial attempts to transplant the canine pancreas with maintenance of exocrine secretion utilized the duodenum as a conduit to the skin (24). The first clinical transplants in humans by Lillehei in 1966 used a variation of this technique (26). Largiader was the first in 1967 to successfully transplant the pancreaticoduodenal allograft with internal drainage via a Roux-en-Y loop of recipient jejunum (37). Several other groups followed using similar techniques to manage the exocrine drainage of the grafts. These early attempts at whole organ transplantation were largely unsuccessful due to technical complications of the procedure relating to the management of the exocrine secretion (37,38,39). Complications included duodenal necrosis, anastomotic dehiscence, fistula formation and thrombosis of the vascular pedicle (37).

Because of the high incidence of complications associated with whole organ grafts, this technique has largely been abandoned in favor of segmental transplantation. This graft consists of the body and tail and has its blood supply from the splenic vessels (38). This technique has the advantage that it is technically easier and it can be taken from a living related donor since more than 50% of the pancreas remains in-situ with the donor.

The problem of managing exocrine secretions still exist however, and a number of techniques have been devised to deal with this. The pancreatic duct can be anastomosed to the ureter (40) or to a Rouy-en-Y jejunal limb (41), however, the same problems with anastomotic leaks, enzyme activation and local necrosis can occur (24). Ligation of the

pancreatic duct leads to local complications including pancreatic ascites and ultimately, chronic fibrosis and loss of endocrine function (24,37). Kyriakides demonstrated the feasibility of allowing the pancreatic duct to drain freely into the peritoneal cavity (42). In the absence of enterokinase the exocrine enzymes are not activated. Intractable pancreatic ascites, has been of problem with this technique however. More recently, Dubernard and associates, in Lyon, France have been injecting the polymer neoprene (43) into the pancreatic duct, this not only occludes the duct, it also suppresses pancreatic secretion. Although questions still exist as to the long-term effects of these polymers on endocrine function, the use of duct obliterated segmental grafts has been one of the more successful of the various techniques of whole pancreas transplantation (37).

From December 1966 through October 1986, 1001 whole organ transplants have been performed clinically (44). Of these, 425 have been performed since July 1, 1977. Currently 329 patients have functioning grafts, 160 for more than one year and 16 more than 4 years. The one-year actuarial patient and graft survival rates (insulin independent) since 1985 are 83% and 44% respectively. Although the survival rates recently are significantly higher than previous years these figures are reflective of the problems that still exist with immediately vascularized grafts. Whole organ pancreas transplantation has not been applied to the treatment of large numbers of diabetics. Organ procurement and storage are still major obstacles as well (25).

Technical failure aside, a major issue with pancreatic transplantation is the need for immunosuppression. While ideally pancreatic transplantation would be performed in young diabetics before

the development of microvascular complications, it must still be conceded that pancreatic transplantation is an experimental procedure and that immunosuppression is hazardous (45). Moreover, the endocrine pancreas cannot be regarded as an immediately life sustaining organ. For these reasons, present indications for clinical use of this procedure are restricted almost exclusively to selected diabetic patients already requiring immunosuppression for maintenance of a coexistent renal allograft (25,37,45,46).

In summary, major problems with whole organ or segmental pancreatic grafts include, technical difficulty in performing the transplant, management of the exocrine secretions, requirement for immunosuppression and the problem of organ storage. Theoretical advantages exist for all four of these fundamental problems, when considering free grafts of pancreatic islets as an alternative form of transplantation (37,47).

The ease of the technique of transplanting islet cell preparations is attested to by the number of researchers who have performed this experimental procedure successfully (24,37). Management of the pancreatic duct is no longer an issue with islet transplantation. Immunologic manipulation of the islet cell graft to reduce immunogenicity has been documented (48). Finally, the ability to store islet cell grafts by cryopreservation prior to successful transplantation has been demonstrated experimentally (49).

Islet Cell Transplantation

The problems associated with presence of exocrine tissue in whole pancreas grafts has prompted investigation of the effects of isolated islet transplants in many laboratories. From a theoretical point of view, transplantation of normal beta cells into an insulin deficient,

diabetic recipient is the most logical approach to treatment of the disease (25).

The earliest attempts at islet cell transplantation were free grafts of pancreatic fragments. In 1929, Brancati, succeeded in autotransplanting free segments of duct-ligated canine pancreas into the greater omentum (35). Histological examination of grafts that had survived over 30 days showed normal islets among atrophied acini. Classic studies by Browning and Resnick, in 1951, documented short-term histologic and functional survival of subcutaneous allografts of minced murine pancreas (25). In 1959, Brooks and Gifford autotransplanted vascularized canine pancreas into the rectus muscle. The grafts were then allotransplanted as large fragments but did not function (35). These initial attempts to transplant pancreatic fragments were considered unsuccessful and unsafe because the associated exocrine enzymes autodigested the transplant tissue and injured the host (50).

The detrimental effect of transplanted acinar tissue on graft function and viab [illegible] encouraged the development of islet-rich tissue. Most of the pioneering work attempting to produce relatively pure islet preparations was done using the rodent model. Two phases marked the development of islet isolation. Microdissection began with Bensley who studied islets within the guinea-pig pancreas and picked them free from acinar tissue (35). Microdissection techniques, however, were traumatic, and produced only small numbers of islets.

It was the failure of microdissection and the need for islets to be used for in vitro studies that prompted Moskalewski's work in 1965 (51). He was the first to describe the enzymatic method of islet isolation using the enzyme complex collagenase. Although the enzy[illegible]e destroyed

many islets, it did allow complete separation of islets from acini. The islets were viable and responded to in vitro glucose stimulation by degranulation. Lacy and Kostianovsky improved the technique by intraductal distension of the pancreas before digestion and separated islets from the digested pancreas by centrifugation in sucrose gradients (52). These modifications resulted in the isolation of approximately 300 intact islets from a single rat pancreas. More efficient separation was reported by Sorenson using density gradients of Ficoll (53). Following Ficoll centrifugation the islets could then be hand picked to provide a relatively pure preparation of islets. Suspending the Ficoll separated islets in phenol red solution under a dissecting microscope with a reflected green light facilitated the process of hand picking (54). Subsequently, Lacy and associates assessed the viability of isolated islets in vitro by perifusion demonstrating that islets placed on a millipore filter within a plastic chamber responded to glucose stimulation with a biphasic secretion of insulin (55).

The gradual improvement in islet isolation techniques eventually provided the ability to carry out transplantation experiments using relatively pure isolated rodent islets. Younoszai et al. in 1970, first reported the transplantation of isolated islets (56). They temporarily ameliorated chemically induced diabetes in the rat by intraperitoneal transplantation of isolated islets.

The first major exercise in experimental islet transplantation was reported by Ballinger and Lacy in 1972 (57). Intraperitoneal or intramuscular isotransplantation of 400-600 isolated islets into streptozotocin-diabetic rats, produced long-term amelioration of hyperglycemia, polyuria and glycosuria. Excision of the intramuscular

transplants restored the diabetic state. Their work was independently confirmed by Reckard and Barker the following year (58).

A significant advance in experimental islet transplantation was reported by Kemp who, in 1973, described a method of implantation that improved the efficacy of islet transplantation without increasing islet requirements (59). Intraportal embolization of only 400-600 rodent islets completely normalized blood and urine glucose levels, where as the same number of islets transplanted intraperitoneally only ameliorated the diabetic status of recipients. Intraportal embolization probably owes its success to increased islet viability resulting from an immediately available blood supply and a physiological route for insulin secretion. The importance of the portal circulation for insulin activity has been demonstrated (23).

Since Kemp's work many groups have successfully applied intraportal injections of islets for long-term reversal of diabetes in rodents (24). The number of islets required for successful transplantation depends on several factors including the integrity of the islets and the severity of the preexisting diabetic state. As few as 240 or over 2000 islets may be required to restore normoglycemia in rats. The latent period between transplantation and amelioration of diabetes is shortened, and glucose tolerance improved as larger numbers of islets are used. If over 1000 islets are used normoglycemia reliably ensues within 1-2 days. If only 200-400 islets are isolated from a pancreas multiple donors may be needed. In rodents this is not a problem since syngeneic strains are available for experimental studies (24).

Besides the liver and peritoneal cavity isolated islets have been transplanted to a multitude of sites in rodents with variable results.

Intrasplenic injection is nearly as efficient as intraportal injection in rats (24). Islet cell function and morphological viability have been documented following embolization to the lung via a systemic vein, direct injection into the liver, implantation under the kidney capsule,intracerebral inoculation, transplantation to an omental pouch, or anterior chamber of the eye, as well as subcutaneous, intratesticular, intrapancreatic or intrasalivary sites (24,25,60). Reversal of experimental diabetes was most reproducible with intraperitoneal and intraportal islet grafting (25).

An alternative approach to obtaining an islet rich tissue has been the use of fetal and neonatal pancreatic tissue. The rationale for using these tissues is based upon the variable rates of differentiation between exocrine and endocrine components of the pancreas. Endocrine cells appear early in organogenesis and are able to synthesize insulin and glucagon at a time when acinar cells are undifferentiated. Furthermore, endocrine cells have a high growth potential and the choice of an appropriately aged fetal or neonatal pancreas will provide islet rich tissue that may continue to develop without the potential for autolysis (37).

Leonard and Lazarow first demonstrated that diabetes in rats could be ameliorated by intraperitoneal transplantation of multiple neonatal pancreases dispersed by collagenase digestion without specific islet isolation (61). Subsequent investigators have shown that diabetic states in rodents can be reversed by intraportal, intrasplenic, or intravenous administration of dispersed neonatal pancreas (24). The limiting factor in the use of neonatal tissue is the large number of donors required to effect rapid reversal of diabetes. Other researchers

have demonstrated the use of fetal tissue to reverse the diabetic state as well (62,63,64).

The results of the above series of investigations, as well as others, have established that complete reversal of diabetes in rodents is possible by transplantation of islet tissue. Extensive metabolic studies have demonstrated normalization of growth, glucose tolerance and a variety of other metabolic factors (25,65). Islet transplantation in rodents was demonstrated to halt and reverse the microvascular changes of diabetes (16,29,30).

The early success of islet transplantation in rodents prompted studies in larger animals. It was quickly realized that the procedures for isolation developed using the rodent model would not be adequate in dealing with the more compact and fibrous pancreas of higher mammals (24,25,66) The yield of islets from one donor was insufficient to reverse the diabetic state. Unlike rodents, multiple allogeneic donors could not be used as islets would be subject to rejection before function of a technically successful graft could be determined.

The problem of islet yield was partially solved by Mirkovitch and Campiche (67). They reversed diabetes in totally pancreatectomized dogs by intrasplenic autotransplantation of dispersed pancreatic tissue prepared from the pancreas by collagenase digestion alone, with no attempt at islet purification. Splenectomy lead to severe hyperglycemia and death. They chose the spleen because of its rich blood supply with venous outflow to the portal circulation.

The work of Kretschmer and associates confirmed the feasibility of using collagenase dispersed fragments without purification to produce normoglycemia in pancreatectomized dogs (68). Using control dogs they

demonstrated the necessity of collagenase digestion during graft preparation for successful islet transplantation. Other investigators have successfully used the model of intrasplenic transplantation of islets (24).

The technique described by Mirkovitch and also by Kretschmer involves direct inoculation of the islet cell graft into the splenic pulp. Warnock et al. have modified the procedure. They have successfully produced normoglycemia in pancreatectomized dogs by refluxing the graft in retrograde fashion into terminal splenic veins (69). They demonstrated superior function when compared to direct splenic inoculation.

While embolization of pancreatic islets to the liver via the portal vein has been shown to be the optimal method of transplantation in rodents (59), it is not the case in the canine model. Diabetes has been treated by infusion of collagenase dispersed pancreatic tissue into the portal vein of pancreatectomized dogs (70,71). Serum liver enzymes are transiently elevated and the dogs are particularly susceptible to the development of portal hypertension. Kretschmer (71), found this route to be less effective than transplantation to the spleen. Mehigan described disseminated intravascular coagulation and portal hypertension after intraportal transplantation (72). This syndrome was secondary to the release of thromboplastins in the preparation and could be prevented by prophylactic administration of heparin and aprotinin. Lorenz has transplanted collagenase digested islets purified by Ficoll gradient separation via portal embolization. The dogs failed to develop enzyme abnormalities or portal hypertension (73). The intraportal route has also been used successfully for pancreatic islet transplantation in

Baboons (74), rhesus monkeys (75), and pigs (76). With respect to heterologous sites of transplantation successful engraftment of islets in a renal subcapsular position has been reported (77). The superiority of the splenic bed as a transplantation site for pancreatic fragments in dogs has been emphasized by several investigators (67,69,71).

The above work demonstrates that, by eliminating the steps of purification, sufficient islet tissue can be obtained from one donor to produce long-term normoglycemia in the canine model.

According to the pancreas and islet transplant registry there have been a total of 79 cases of islet autotransplantation after total or near total pancreatectomy performed world wide (78). Approximately one-half of the 79 recipients were reported to be insulin independent after the procedure. The results are difficult to interpret and the relative contribution of the transplanted islets or the pancreatic remnant to maintaining glucose homeostasis cannot be discerned.

There have been a total of 166 allotransplants of pancreatic islet tissue performed throughout the world to date (78,79). The islet preparation techniques, the sites of transplantation, and the application of immunosuppression have been extremely variable in the islet allograft attempts. There have been no reports of transplantation of truly purified human pancreatic islets. Currently, none of the recipients of free pancreatic islet allografts is insulin independent. It appears that a method of islet allotransplantation suitable for clinical application is not yet available.

Despite the current lack of success at transplanting islets in the clinical setting, research over the last few years has demonstrated that the concept of transplanting insulin producing tissue in the diabetic

patient is a goal that can be achieved. A tremendous amount of research is currently approaching the problem of increasing both the yield and the purity of islet cell grafts (66).

At the same time a number of laboratories are applying efforts toward solving the ultimate problem facing pancreatic transplantation, the rejection process (48,66). The use of immunosuppression is being studied but the ability to immuno-alter the graft prior to transplantation or to immuno-isolate the graft may ultimately allow allotransplantation of pancreatic islet tissue without the use of suppressive drugs and the fear of rejection (48).

Further studies are also needed to delineate the ideal site for transplantation of pancreatic islets. Such a site should provide a rapid vascular supply for the islets, autologous venous drainage to the liver of their released hormones and easy access both for transplantation and removal of islet tissue (66). Which site will best meet these criteria remains to be determined.

Approaching the optimal islet preparation, defining the most efficient and safest site, and manipulating the tissue appropriately to prevent rejection should confirm whether islet tissue transplantation will be the best way to offer diabetic patients a form of therapy that may effectively prevent the complications of their disease.

Current Issues in Islet Cell Transplantation

i) Increasing Islet Yield

Merkovitch and Campiche were the first to demonstrate amelioration of diabetes in the canine model with islet-containing tissue from 1 donor (67). This was done by transplanting pancreatic fragments prepared by collagenase digestion and mechanical dissociation,

eliminating the steps used for graft purification used in the rodent model. The above study, however, and subsequent experiments (68,80) has demonstrated that metabolic function in diabetic recipients of pancreatic fragments was only partially restored. As a consequence, different aspects of the islet isolation process have been examined and modified in an attempt to improve islet yield.

The very nature of the techniques described previously are partially responsible for the difficulty in isolating islets. Ductal distention and disruption expose interlobular planes to collagenase which digests exocrine and endocrine tissue indiscriminately (81). Mincing the pancreas results in 50% loss of tissue insulin (67). Incubation of the chopped pancreatic fragments in collagenase destroys more than 70% of the islets as well as the acinar tissue (82). The yield of islets obtained by mechanical dispersion and enzymatic digestion of the pancreas was less than 10% of the total islet mass of the original gland (83).

Techniques for improving islet yield currently under investigation can be divided into 4 major categories: alterations to the pancreas in vivo, manipulations to the pancreas in vitro, assessment of the technique of pancreatic dispersion and most importantly, a reexamination of the method of digestion of the pancreas (66).

While the pancreas is still in the donor there are several manipulations that may improve islet yield. Payne (84) demonstrated that administration of DL-Ethionine to rats prior to isolation improves islet yield. DL-Ethionine selectively destroys acinar cells without effecting islets, however, the technique required prolonged administration prior to gland harvesting, an impractical procedure with

cadaverous donors, and as well DL-Ethionine is a known carcinogen. The use of pilocarpine as a pretreatment in a similar fashion has also demonstrated an increase in islet yield in rats (85). Scharp has examined the addition of pilocarpine, pancreozymin and secretin to the ductal perfusate and has increased islet yield (66).

The next opportunity for modifying the isolation process comes with removal of the intact pancreas. Lacy (52) has shown that mechanical distension of the rodent pancreas increases islet yield, by causing mechanical separation of islets from exocrine tissue making digestion easier. Ductal distension does not work as well with the more fibrous canine and human pancreas (66). Alternative methods of distending the gland have been examined. Intra-arterial distension was unsuccessful as the islets were essentially exploded by the pressure (66). Downing and co-workers demonstrated an increase in islet yield by distending the dog pancreas through the venous system of the gland rather than the duct (86). Combining the technique of pancreatic distension with digestion by incubation still did not produce sufficient free islets to permit single donor transplants in dogs. The next advance in treating the pancreas in vitro came from Horaguchi and Merrel (83) when they introduced the concept of retrograde perfusion of the gland with collagenase prior to islet isolation. With this technique, in the canine model, a 57% recovery of beta cell mass was suggested by insulin recovery. A 6 fold increase in beta cell content compared to the intact gland was noted. Normoglycemia in dogs was noted following transplants to the spleen and liver. The importance of this step has been demonstrated by others since (66,69).

The method of mechanical dispersion of the gland used has received less attention than other aspects of the isolation process. Some favor simple cutting of the gland with scissors (66), while others prefer chopping with counter rotating blades (69). Gray and co-workers recently described a modification of the digestion-filtration process that they have applied to the human pancreas (87). They emphasize that their method does not involve mechanical chopping of the gland. The gland is digested by intraductal distension with collagenase and cut into fragments with scissors. The fragments are then teased apart releasing numerous fine fragments into cold Hank's solution. Further dispersing of these fragments was performed by gently passing them through 14 and 15 gauge needles. Using this method 1,101 islets per gram of tissue were isolated. Collagenase isolation of islets has received the greatest attention from islet investigators. Most researchers use a technique based on Moskalewski's original method (51). The method destroys islets that are released early in the digestion process while at the same time leaves much of the islet mass trapped in the pancreatic fragments. It is extremely inefficient (81). Scharp has approached this problem and developed a digestion perfusion technique that eliminates the need to identify a set end point (75). Chopped, pancreatic fragments were loaded into a single sterile screen and a series of separate digestions were performed washing the released islets out of the chamber. Further examination of the initial digestion-filtration process led to modifications designed to increase the efficiency or convert the process from discontinuous to a continuous digestion-filtration process. The result of this effort is the auto-isolator (66). The device has fine stainless steel screens over

which the pancreatic fragments are placed. Enzyme is then pumped into the screens and across the tissue fragments. Scharp has demonstrated that using this method that over 90% of single donor, single recipient islet autotransplants in dogs have been successful.

Lacy and associates have evaluated a novel way to retain the pancreatic fragments while permitting collagenase to release islets (88). They have used the universal fastener "Velcro" to hold onto the fibrous portion of the gland. this method seems to yield more intact islets than the auto-isolator (66).

On the basis that collagenase destroys islets and contaminates the graft investigators have evaluated the feasibility of preparing islet rich pancreatic fragments by eliminating enzymatic digestion completely. Hinshaw and associates (89) reported isolation of 500,000 - 2,000,000 islets from a single human pancreas by hand pressing undigested pancreatic fragments through a stainless steel screen 200-280 μ pore size. Toledo-Pereyra (77) reports normoglycemia in dogs allotransplanted with non-collagenase digested tissue prepared by pressing pancreatic tissue through a steel screen with 1.5 mm pore size. However, the necessity of the collagenase digestion step in islet isolation has been documented by others (68).

In general, the methods being evaluated to isolate islets in large animals and man provide a sufficient number of islets to achieve normoglycemia,as shown in dogs, but the islets are so few in number or so contaminated with exocrine cells that they are risky for clinical trials (90).

ii) Increasing Islet Purity

Increasing the purity of islet cell grafts is important from the point of view of safety as well as for immunological regions. Doing away with unwanted exocrine tissue will avoid the problems associated with this tissue and production of a purer graft may produce greater success with allotransplantation.

Although current interest in tissue culture is as a means of immuno-alteration this technique was initially examined as a means of graft purification. Lazarow et al (91) showed that culture of human fetal pancreas resulted in disappearance of acinar cells and enzyme production while islet cell mass was preserved. Similar findings have been demonstrated in the rat (92), the dog (92,93) and the human (94,95). Acinar cells are autodigested due to the action of intrinsic enzymes while the islets are relatively spared resulting in purification of the tissue.

Matas (93) has demonstrated reversal of hyperglycemia in dogs rendered diabetic by partial pancreatectomy and streptozotocin transplanted with pancreatic fragments cultured for 24 hours. Insulin content of the cultured tissue was reduced by over 1/2 compared to fresh tissue, however. Another approach to purification of islet cells is the production of pseudo islets. This technique, is being investigated by Scharp and associates (66), involves the digestion of pancreatic tissue with collagenase and trypsin to a single cell stage. Single islet cells are then separated from contaminating exocrine tissue initially by use of Ficoll sedimentation and later with the Beckman Elutriator. The elutriator works on the principal that counter-current centrifugation separates particles of different size (96). Gyrorotational culture is

then used to re-aggregate the purified single cells into pseudo-islets. Scharp has shown by immunoperoxidase staining that the pseudoislets are comprised of over 90% islet cells which release insulin in vitro. The process has been adapted to be used with the auto-isolator, however, in the dog model only 25% of autotransplants became normoglycemic. Scharp has concluded that this process results in very pure islet tissue but gives a marginal yield (66).

Several other means of islet tissue purification are currently being investigated. Scharp and associates have been examining a new electrophoresis device developed by the McDonnell Douglas Corporation. Preliminary trials have demonstrated the feasibility of partially separating individual islet cell types from each other. The fluorescent activated cell sorter has been shown to be able to partially purify islet cells. Another approach in purifying islet tissue is the use of monoclonal antibodies directed against islet tissue. The cell preparation to be purified is either passed through a column or incubated in petri dishes. The antibody complexes with the appropriate cell types and retains them. Further elutriation then releases the desired cells in a purified form (66).

Nason and associates recently described the effect of high dose radiation on pancreatic microfragments (97). Subjecting the islet cell graft to 5000 rads resulted in destruction of contaminating exocrine cells as documented by electron microscopy. This resulted in purification of the graft as suggested by a 2 to 3 fold increase in the insulin/amylase ratio of the graft following irradiation. Viability of the irradiated graft was supported by in vitro challenge with glucose

and a 70% success rate at 1 month in dogs autotransplanted to the spleen.

A technique of canine pancreatic islet isolation which results in highly purified islets has been described by Noel, Alejandro and associates (98,99). Their technique involves initial distension of the gland with an enzyme solution of collagenase and DNase. The left and right limbs are distended through the duct. The tissue is then incubated at 37°C. The gland is then distended with a solution of hypertonic EGTA and sucrose and again incubated and this is followed by a second digestion with the enzymes. Simple agitation is used to dissociate the pancreatic tissue from remaining undigested tissue, ducts and vessels. Further dispersion is produced by trituration of the pancreatic tissue with 14, 16 and 20 gauge needles attached to a syringe. This material is then separated using discontinuous Ficoll centrifugation. Islet tissue is removed from 3 distinct layers and then incubated in a complement solution which causes disruption of contaminating exocrine cells. The islets are then cultured overnight prior to transplantation. They state that the tissue is highly enriched for endocrine cells determined by cell counts from electron microscopy, demonstrating an increased percentage of endocrine cells from 1-3% to 60-84% in the final graft and a graft volume of only 1.5 cc. Alejandro successfully induced normoglycemia in pancreatectomized Beagles in 15 of 24 dogs autografted to the liver via portal embolization for periods of up to 20 months (98). More recently Warnock and Rajotte have developed a technique for reliably isolating highly purified canine islets (100). They combine the principles of collagenase perfusion of the ducts, gentle dissociation of tissue, and density gradient separation to

isolate purified islets. Normoglycemia was successfully induced in pancreatectomized dogs following auto-implantation of purified islets into both the spleen and liver.

iii) Increasing Allograft Survival

The ultimate problem facing pancreatic islet cell transplantation is allograft rejection. It was initially hoped that islet cell grafts would be weakly immunogenic. This hope was based on prior observations that other endocrine tissue such as parathyroid and ovary evoked only a feeble immunologic response when transplanted (39). However, the earliest transplantation of allogenic islets demonstrated that they were rejected very rapidly, surviving only a few days in the rat (101). In 19 separate studies cited by Sutherland (24), rat islets transplanted across a major histocompatibility barrier survived as long as 6 days only in 2 studies. Minimizing histoincompatibility by using major histocompatibility complex (MHC) compatible donors fails to extend the survival of rat islets allografts beyond 8 days (39). Various types of specific immunosuppression have been used in islet transplant experiments. Prednisone, azathioprine, cyclophosphamide and cyclosporine A have all proved relatively ineffective in prolonging survival of isolated islet allografts (39). Even antilymphocyte serum, which is the most effective immunosuppressive agent in prolonging islet allograft survival in rats, is more effective in extending skin and vascularized allograft survival. Islet cell grafts have been transplanted to "immunoprivileged" sites as well. The anterior chamber of the eye, the hamster cheek pouch, the testes and the renal subcapsular region have been used. The use of immunoprivileged sites has not consistently led to prolonged allograft survival. It would

therefore seem that islet cell grafts are particularly susceptible to rejection. The reasons for this are not known. A possible explanation of the very brief survival of islet allografts is that like other dissociated cell grafts (eg. bone marrow) they may be susceptible to damage by humoral immunity (39). Islet allografts established in allogenic but tolerant hosts remain vulnerable to destruction by administration of antibody whereas allografts of vascularized pancreas are not damaged (102).

Another explanation is based on the fact that quantitatively less insulin secreting tissue is engrafted with islet cell transplants than whole organ grafts. If the number of islet transplanted is just sufficient to normalize hyperglycemia the destruction of a few islets would result in a return to the diabetic state. Finch and Morris have demonstrated that by increasing the number of transplanted islets functional survival of islet allografts in rats can be prolonged (103).

Nevertheless, recent experimental findings suggest that despite the highly vulnerable nature of islet tissue allografts, the islets themselves may be only weakly immunogenic. The evidence for this contradictory situation comes from experiments in which pretransplant treatment of islets has been carried out to ensure implantation of pure endocrine tissue (104,105).

Lafferty and associates stimulated this work when they demonstrated that thyroid allograft survival was prolonged when the tissue was cultured in 95% O_2 (for 3-4 weeks prior to transplantation (106). Lacy subsequently adapted the technique for pancreatic islet tissue (104). High O_2 tensions are toxic to islet cells, however, Lacy demonstrated that rat islets cultured at 24°C for 7 days could be allotransplanted

into recipients receiving a single dose of antilymphocyte serum with 85% survival of grafts at 100 days. Subsequently it has been shown that pretransplant culture alone is capable of extending survival of islet allografts (105).

The rationale for improved results with allografts following pretreatment by culture is based on the "passenger leukocyte" theory first proposed by Snell in 1957. Lafferty has recently summarized this theory and elaborated on how it applies to modern concepts of immunoregulation and immuno-alteration prior to transplantation (107). The theory holds that a small number of allogenic leukocytes could stimulate a strong immune reaction. It is possible that the host sensitization resulting from islet tissue grafts is actually caused by the passenger leukocytes contaminating the graft rather than the endocrine cells themselves.

This premise was substantiated by demonstrating the acute rejection of cultured islet cell grafts when recipients were injected with donor peritoneal exudate cells, a lymphoid cell population enriched with macrophages (108). The donor lymphoid cells initiated host recognition of the foreign or transplant antigens of the graft which served as targets for immune rejection. Thus even though cultured islets still appeared to express foreign transplant antigens, an immune rejection response was not initiated without donor lymphocytes.

Further investigation established that the islets might in fact be deficient in certain histocompatibility antigens. Two functional groups of antigens coded by the major histocompatibility locus have been examined by various techniques. Class I antigens (H-2K and H-2D in rodents, HLA-A, HLA-B and HLA-C in humans) are believed to be present on

most nucleated cells and are target antigens in transplantation rejection. In contrast Class II antigens (Ia in rodents; HLA-DR in humans) are believed to control cell-cell interactions and the proliferative response of the recipients lymphocytes that ultimately destroy the graft. Parr (109) using an immunoferritin labeling technique was unable to demonstrate any H-2 antigens on the surface of beta cells of dissociated mouse islets while high concentrations of H-2 antigen were found on acinar, ductal and capillary endothelium. Faustman and associates studying rodent islet cells with cytotoxicity and absorption assays found that H-2Da nd H-DK antigens were present on islet cells, however, no Ia antigens could be demonstrated (110). Subsequent studies of mouse, rat and human islets have confirmed that Class I antigens are present but Class II antigens are lacking on islet cells (111). Hart and co-workers (112) used monoclonal antibodies to identify Class I and Class II antigens on rat pancreas and pancreatic islet cells, or fixed tissue macrophages stained intensely for Class II antigens. They postulated that the immunogenic dendritic cell represented the immunogenic passenger leukocyte.

At the present time it is generally felt that islet cell grafts, depleted of passenger leukocytes by a period of tissue culture can be allografted presenting only Class I antigens to the recipient. Having been depleted of Class II antigens necessary to provoke an immune response the transplanted islets would not be rejected even by nonimmunosuppressed recipients. However, they would be rejected if the recipient is confronted with donor Class II antigens at some point before or after the islet transplant.

Complicating this possibly simplified view is the finding of class II antigens in the surface of rat endocrine cells by Ulrichs, and Muller-Ruckholtz (113). They used monoclonal antibodies to demonstrate that 20-40% of islet beta cells expressed Ia, although to a weaker degree than macrophages, dendritic-like cells or endothelial cells. They suggest that the passenger leukocyte concept may thus be too simple an approach to the problem of islet immunogenicity.

Presently, there are 4 broad areas in which most current research, looking to prolong pancreatic islet allograft survival falls; graft immuno-alteration, induction of host tolerance, immuno-isolation and immunosuppression.

A) Immunoalteration: The majority of these studies have been in the area of immuno-alteration, extensions of the original work of Lafferty and Lacy in an attempt to prolong allograft survival without the use of immunosuppression.

Lacy originally demonstrated that in vitro culture of donor rat islets at 24°C for 7 days in conjunction with a single injection of anti-lymphocyte serum into diabetic recipients resulted in a 90-100% survival rate at 100 days following transplantation (104). Several other techniques of immuno-alteration have since been described. Bowen et al demonstrated that isolated mouse islets will form aggregates of 50 to form a large megaislet and, unlike isolated islets, would withstand exposure to 95% O_2 for up to 7 days. This pretreatment prevented rejection of islet aggregates when transplanted across a major histocompatibility barrier in mice (114). Lacy and associates subsequently showed that rat islets would also form megaislets and similarity would withstand exposure to 95% O_2. Transplants of rat

megaislets cultured for 7 days in 95% O_2 beneath the renal capsule of diabetic mice resulted in 40-50% survival rate at 70-90 days (115). Other techniques complementing tissue culture have also been developed to immuno-alter islet cell grafts. Faustman and coworkers have used monoclonal antibody against Ia positive donor cells. Incubation of donor mouse islets with Ia antibody followed by complement would produce a 100% survival rate at 200 days when transplanted across a major histocompatibility barrier (116). Hardy has similarly reduced allograft immunogenicity by pretreatment with ultraviolet radiation. He has shown that irradiation combined with 24 hours of tissue culture could indefinitely prolong islet allograft survival in rats (117).

B) Induction of tolerance: Inducing a state of recipient tolerance is also being investigated as a method of increasing allograft survival. It has been demonstrated that transplantation of Ia-negative tissue results in the production of a tolerant state in the recipient (105). Faustman has shown this to be an effective method of prolonging allograft survival by pretreating recipient mice with donor blood depleted of Ia tissue by treatment with monoclonal Ia antibody (118). This pre-immunization regimen resulted in an 85% survival rate of the untreated islet allografts at 100 days (122). Hardy has had similar success pretreating recipient rats with blood similarly depleted of passenger leukocytes by exposure to ultraviolet radiation. He demonstrated 100% survival at 100 days following transplantation of untreated rat islets across a major histocompatibility barrier (119). Another technique of inducing donor tolerance has been demonstrated by Gray et al (120). They showed that rats became tolerant to renal allografts after a 14 day course of cyclosporine A. Long-term

acceptance of the renal graft was followed by an islet allotransplant from the same donor, with 4 of the 5 animals accepting the islets without further immunosuppression. Gray concluded that once a recipient rat has accepted a renal allograft under the influence of cyclosporine, it will accept permanently an islet allograft of the same strain.

C) Immunoisolation: The subject of immuno-isolation techniques as a method of protecting pancreatic islet allografts has been reviewed by Scharp and associates recently (121). The concept of immuno-isolation was developed by Algire, Prehn and Weaver in the 1950's as a means of studying the cellular mechanisms of rejection. They developed the first diffusion chambers to investigate whether cellular or humoral factors were responsible for the destruction of non-vascularized grafts. They used a diffusion chamber with different membranes, which either permitted or failed to permit the migration of host immunocells to the grafts. Membranes that prevented cellular contact resulted in prolonged survival of allogenic tissue. This definitive work clearly established the concept of immuno-isolation and its potential for protecting transplanted tissue from rejection. Subsequently a number of techniques have been studied as methods of immuno-isolation.

Currently there are 4 major techniques being developed to achieve this goal, extravascular diffusion chambers, intravascular diffusion chambers, intravascular ultrafiltration chambers and micro-encapsulation techniques. Each of these major approaches has definite advantages and disadvantages, however the application of these techniques into functional and practical devices will require the solution of many biotechnical problems. Extravascular diffusion chambers can be implanted in many locations but are limited by host fibroblastic

responses which further reduce diffusion capability. Intravascular diffusion chambers have improved diffusion characteristics but have serious vascular access problems. Intravascular ultrafiltration chambers have a more rapid response since they do not rely on diffusion but have an additional problem of protein deposition on membranes micro encapsulation (122) incorporates the islets inside a biochemical membrane making it more efficient but the technique is limited by membrane toxicity and instability.

Immuno-isolation offers an alternative to other methods of improving allograft survival but the technology must be improved to develop suitable materials for effective clinical trials (121).

D) Immunosuppression: The third method of improving islet tissue allotransplantation is the use of generalized immunosuppression and a variety of drugs and protocols have been tested in the various islet transplant models.

Silicca and Carrageenan have been employed due to their ability to inhibit macrophage activity,but only silica was found to be effective preventing rat islet allograft rejection indefinitely if begun 6 days prior to transplant (123). These agents, however, are permanently toxic to macrophages and have not been tried in man.

Most of the immunosuppressive regimens currently used, utilize lymphocytopenic drugs in an attempt to inactivate the cells involved in initiating and effecting the immune response. Chemotherapeutic agents including melphalan, 6 mercaptopurine, azathioprine, methotrexate and cyclophosphamide have been tried.

Cyclophosphamide has shown little effect in pancreatic islet transplants (24). Azathioprine in combination with prednisone has been

the backbone of clinical immunosuppression for over 2 decades. However, results in canine and human whole organ pancreatic transplants and islet transplantation have been poor. Bell et al were unable to prolong normoglycemia in rat islet transplants with azathioprine with or without steroids (24), nor could Kolb and associates in dogs (124). Kretschmer et al using azathioprine and prednisone in canine pancreatic fragment allotransplantation did achieve normoglycemia in 8 of 24 dogs (125). All 8 subsequently died of infections associated with excessive immunosuppression. Even corticosteroids have failed to prevent or reverse islet allograft rejection in rats alone or in combination with azathioprine (24).

Slight prolongation of intrasplenic islet allograft survival has been reported in dogs treate ith azathioprine and prednisone (24), but Kolb (124) et al observed almost no effect with a similar regimen after intraportal islet transplantation in dogs.

Antilymphocyte serum on one of its derivatives have been the most effective agents in delaying rejection of islet allografts in animals (24). Frangipane (126) showed prolongation of mouse islet allograft survival and Beyer (127) prolonged allograft survival in rats. Barker et al demonstrated increased survival of xenografts from rat to mouse with antilymphocyte serum (128). Lorenz and co-workers extended duration of normoglycemia from [illegible] months to greater than one year in dogs combining antilymphocyte globulin and azathioprine in dogs with weak histoincompatibility (129).

Cyclosporine A has proven to be a potent immunosuppressive agent in most experimental models of tissue transplantation as well as clinical transplantation of kidneys, bone marrow art and liver. It is of

particular interest in pancreatic transplantation because of its potential use in a single agent thus reducing the need to use steroids with their undoubted diabetogenic activity (130). However, a variety of experiments have thus far shown cyclosporine to be less successful in preventing rejection of pancreas allografts in experimental animals than with other tissues. In whole organ allografts, cyclosporine has been shown to prolong survival in rats (131). Cyclosporine in doses of 10 mg/kg per day for 15 days showed modest prolongation of survival while raising the dosage to 20 mg/kg increased average survival slightly. A dosage of 40 mg/kg was found to be toxic, all animals dying while receiving cyclosporine. Rynasiewicz and colleagues achieved better results using continuous administration of cyclosporine (132). Using dosages of 10 mg/kg daily, 57% of segmental grafts were not rejected when transplanted across a minor histocompatibility barrier. Higher doses presented rejection completely but infective complications resulted in death in 50% of cases.

In dogs, McMaster and associates found rejection of duct occluded pancreas allografts was delayed by continuous oral cyclosporine administration (133). Untreated dogs remained normoglycemic for a mean of 13 days while those receiving 25 mg/kg per day cyclosporine were normoglycemic for a mean of 85 days.

Work by DuToit and Garvey in Oxford support the modest prolongation in whole organ graft survival with the use of cyclosporine, although Garvey attributes the poor results to inadequate absorption of the orally administered drug in pancreatectomized dogs (130).

Results with cyclosporine in prolongation of a graft survival with dispersed pancreatic islet preparations h generally been

unimpressive as well. The survival of intraportal islet allografts transplanted across a major histocompatibility barrier was unchanged with doses of cyclosporine up to 20 mg/kg/day given for 14 days and even doses as high as 40 mg/kg/day produced only a mean survival of 10.3 days with several rats dying of toxic effects (131). Rynasiewicz also showed no prolongation of islet cell survival in rats transplanted across a major histocompatibility barrier with doses up to 50 mg/kg/day (134). Others have demonstrated significant prolongation of islet cell graft survival in rats with cyclosporine, with high doses (50 mg/kg/day) with comparatively little toxicity (135,136). The rats, however, tend to reject their grafts shortly after the cyclosporine is stopped.

The effect of cyclosporine on allografts of pancreatic microfragments in large mammals has also been examined, although experience is limited.

DuToit and associates treated dogs allografted with cyclosporine for 14 days. Although the dogs never became normoglycemic survival time was prolonged in comparison to allografted dogs without treatment (130). Kneteman and associates compared dogs allografted with pancreatic microfragments without immunosuppression with dogs treated with azathioprine and prednisone or cyclosporine A (137). Rejection (plasma glucose > 150 mg/dL) was prolonged from a mean of 1.8 days in the azathioprine/prednisone group to 19.3 days in those treated with cyclosporine. Untreated dogs rejected at a mean of 5.0 days. Similarly survival was prolonged in the cyclosporine group (mean 33.3 days) when compared to the azathioprine/prednisone gp (mean 13 days) and the untreated dogs (mean 16 days). The contrast between the effect of cyclosporine on islet allografts and on other tissues is striking. The

above studies show that cyclosporine can prolong allograft survival but rarely produces long-term survival.

Recently Alejandro and associates demonstrated for the first time significantly improved pancreatic islet allograft survival in a large animals with the use of cyclosporine A (99). They allotransplanted pancreatic islet tissue to 19 pancreatectomized beagles and three spontaneously diabetic dogs. Allografts were from one or more unrelated donors. The islets, enriched and contained in a packed cell volume of less than 1.5 cc were embolized to the liver via a branch of the superior mesenteric veins. Treatment of diabetic recipients with cyclosporine was begun 3-5 days before transplantation and dosage adjusted to keep serum through levels between 400-600 ng/mL. Five dogs with cyclosporine serum levels below 155 ng/mL promptly rejected their grafts. Only 1 of 17 animals with serum levels above 400 ng/mL rejected 30 days post-transplant. Cyclosporine A was discontinued 30, 60 or 90 days after continuous therapy in 10 animals. Graft failure was observed 2 months later in 1 animal and 5 months in another. Eight other islet allograft recipients have sustained fasting normoglycemia for 7 and 8 months in 2 and for at least 2 month in the remainder. Alejandro concluded that short-term cyclosporine therapy prolonged survival of islet allografts and induced a state of immune unresponsiveness to islet cell antigens in Beagles.

Recurrent Diabetes

Another potential barrier to successful pancreatic transplantation is the recurrence of the original disease process in the transplanted tissue. This is a possible outcome in what may be an autoimmune disease. In kidney allografts destruction by recurrent

glomerulonephritis occurs with uncertain frequency (39). It is known, however, that in identical twin kidney transplants, where rejection is excluded by definition, recurrent glomerulonephritis may sometimes result in graft failure.

Sutherland and associates have also reported their experience in two cases of twin to twin transplants of immediately vascularized segmental grafts (138). All 4 donors had no history of diabetes. Segmental grafts of distal hemipancreas were transplanted between twins. All 4 recipients were initially normoglycemic but with a few weeks of transplantation 3 of 4 patients had returned to hyperglycemia. Biopsies performed in these patients supported the view of recurrent diabetes, showing much β cell destruction and evidence of "isletitis". One of the graft recipients was managed post-operatively on an immunosuppressive regimen of azathioprine and continues to be insulin independent at 9 months. Sutherland concludes that the phenomenon noted in his work is apparently a reaction quite separate from generalized rejection (138).

An animal model, the BB rat, also allows examination of the recurrence question. This rat develops a spontaneous onset diabetic syndrome characterized by severe insulin deficiently and isletitis and is considered the animal model most closely resembling human Type 1 diabetes (39). Transplantation of islets have been shown to reverse the diabetic syndrome in these rats. However, when BB islets were transplanted to spontaneous diabetics specifically tolerant of allogenic donors so that rejection was excluded as a cause of transplant failure diabetes recurred and histological examination revealed a heavy mononuclear infiltrate typical of isletitis (139). Fortunately clinical results to date indicate that recurrent disease is not the obligatory

outcome of transplantation in the human. Both the timing of the human transplantations and the use of immunosuppression will diminish the likelihood of loss of graft function due to autoimmunity.

Sites for Islet Transplantation

The ideal site for transplantation of pancreatic islets has yet to be determined. The major objective of evaluating possible transplantation sites is to identify the safest and most efficient location for islet transplantation in humans. Considering the venous drainage of the transplant site divides potential areas into either autologous, draining into the portal circulation or heterologous, draining into the systemic circulation. Potential autologous sites include intraportal, intrasplenic, mesenteric, omental or intrahepatic while heterologous sites include subcutaneous intramuscular, intratesticular, intraperitoneal or the renal capsule (66).

Initial islet transplants were to the peritoneal cavity. However, it has been demonstrated in rats that the intraportal route is the most efficient (23,24). There has been only one definitive study evaluating the effectiveness of autologous over heterologous sites. Brown (23) transplanted fetal pancreatic tissue under the renal capsule and showed only a marginal response. By performing a renal vein to portal vein shunt he made the diabetic rat become normal. Support for the superiority of the portal route for delivering of insulin also comes from the work of Albisser and associates (140). They demonstrated an optimal degree of glycemia, as well as other metabolic parameters, with insulin delivered to the portal vein by means of a pump compared to that attained by peripheral infusion of insulin or autotransplanted segmental

grafts draining into the systemic circulation. Other autologous sites such as mesentery and the omental pouch show initial promise (66,141).

Heterologous sites have been assessed including the testes, the lung, anterior chamber of the eye, the salivary gland and while islet graft function and morphologic viability have been documented, diabetes has rarely been reversed (24,25). Intramuscular and subcutaneous sites have not been effective in islet studies (66).

Autologous and heterologous sites have also been examined in large animals. Infusion of pancreatic microfragments into the portal vein of dogs has successfully reversed hyperglycemia (70,71,73,99). Transient elevation of liver enzymes generally occur and dogs develop significant portal hypertension. Walsh and associates describe the onset of severe portal hypertension necessitating a portosystemic shunt in a patient treated with an intraportal autotransplant of pancreatic microfragments (142). Despite performance of the shunt the patient developed hepatic infarction and subsequently died. The cause of the portal hypertension was attributed to vasoactive substances (bradykinin) within the graft. The intraportal infusion of pancreatic microfragments has also been associated with portal vein thrombosis and bleeding esophageal varices (143).

Mehigan and colleagues described disseminated intravascular coagulation and portal hypertension in dogs following intraportal infusion of pancreatic microfragments (72). Both of these phenomenons were felt to be related to vasoactive substances and thrombogenic materials released from cells during the graft preparation that were subsequently released into the vascular system of the recipient. The severity of both the portal hypertension and the degree of clotting

abnormalities were reduced by the addition of heparin and aprotinin to the graft preparation.

Aprotinin is known to bind and inactivate kallikreins (bradykinin) of man and pig but not of dog (144). It has been shown to be effective in preventing transplant induced portal hypertension in pigs, to be only partially effective in dogs and to be ineffective in monkeys. Heparin, which could be expected to prevent secondary thrombosis, was shown to have little effect on portal hypertension in animal studies (144).

Torres and associates describe the development of systemic hypotension during the infusion of pancreatic microfragments intraportally. They felt this was due to vasoactive substances within the graft and the rate at which the graft was injected (145). Purer preparations of pancreatic islets have been infused into the portal vein in dogs successfully without the development of portal hypertension or other related problems (73,99).

The spleen has been successfully used as a recipient site for pancreatic islets (67-71) and this site seems effective for protecting the recipient from vasoactive substances in the dog model. However, metabolic studies in recipient dogs are not completely normal (68,69). These animals have fasting normoglycemia, but K values during glucose tolerance tests are lower than normal dogs and peripheral vein insulin levels are quite low. The renal subcapsular region has recently been assessed in the dog model. Toledo-Peryra (77), successfully reversed diabetes in dogs allotransplanted with noncollagenase mechanically dispersed pancreatic fragments to the renal subcapsular region. The lack of rejection in the nonimmunosuppresed recipients further suggest that the renal subcapsular region may be an immunoprivileged site in

dogs. In contrast Hesse and Sutherland were unable to demonstrate normoglycemia in 11 dogs autotransplanted with collagenase digested pancreatic microfragments to the renal subcapsular region, suggesting that the renal subcapsule is not an appropriate site for transplantation of dispersed pancreatic tissue (146).

Cryopreservation and Islet Cell Transplantation

Cryopreservation holds great potential for the field of pancreatic islet cell transplantation. As clinical islet cell transplantation becomes a reality there will be an increased demand for the procedure, and a need for a method of graft preservation will be created. Such preservation will allow time for histocompatibility testing and permit transport of islet cell grafts from the location of the donor to that of the recipient. Long-term storage would allow for the development of tissue banks where potential grafts could be accumulated allowing for optimal matching of donor and recipient as well as the combining of more than one suitable graft, should multiple donors prove necessary.

Cryopreservation provides a technique for long-term storage of isolated islets without cumulative loss of function inherent in other storage methods. Alternative storage techniques such as cell culture and simple hypothermia have been used successfully for short-term preservation of islet preparations, but have been limited by a progressive loss of cell viability over time.

Knight et al were the first to report successful hypothermic storage of isolated islets, but secretion of insulin was decreased by 50% within 48 hours (147). Frankel stored mouse islets at 8°C for 5 weeks and was able to retain 2/3 of insulin secretion, but only if islets were rewarmed to 37% weekly for a brief period (148). Shulak

demonstrated satisfactory function of islets stored at 4°C for up to 48 hours after which time the grafts deteriorated (149). Other studies have found a similar time limitation to the success of cold storage (24). Tissue culture can also be used for short-term storage of islet tissue. Anderson restored normoglycemia to diabetic nude mice within 2 weeks by transplantation of 500 islets which had been cultured for 10 days (150). Nakagawara reversed diabetes in rats by transplantation of 7 day cultured isologous islets (151). The use of tissue culture has also been applied to purification (91,92) and immuno-alteration (104,105) of islet cell grafts but their technical success depends upon preservation of viability. Tissue culture techniques have also been used for preservation and purification of pancreatic microfragments in the canine model (93), but transplants to diabetic recipients were only partially successful. In general, the use of tissue culture for storage of islet tissue for over 1 week have not been successful and the relatively sophisticated equipment and fastidious conditions required for maintenance of islets have prevented successful application of the technique to large animal transplantation models.

Cryopreservation, however, can store islet tissue for prolonged periods. Rajotte and Scharp, documented successful function of islets cryopreserved at -196°C for 2 to 5 weeks transported between centers and transplanted in rats (152). In vitro function of islets cryopreserved for 1 1/2 years has been documented by Bank (153).

Cryopreserved pancreatic tissue has been shown to be viable both in vitro and in vivo (154,155). Several groups of investigators have reported viability of cryopreserved fetal and neonatal small-mammal pancreatic preparations. Slow cooling (0.3°C/min) of 17 day fetal rat

pancreata frozen in 2M dimethyl sulfoxide (DMSO) gave 80% survival as judged by the ability of frozen-then-thawed pancreata to incorporate amino acids in protein (156). Although warming and dilution rates had little effect on percent survival, full permeation with cryoprotectant was required and when glycerol was used, permeation had to be at 22°C and for much longer (157). Single frozen-thawed fetal pancreata implanted beneath the kidney capsule can reverse the diabetic state in rats if implanted for 21 days in healthy carriers (158). Neonatal rat pancreatic fragments, cooled slowly to -70°C, permanently normalized diabetic recipients when insulin was given for 1-2 weeks after implantation (159). Human fetal pancreata have been viable after cryopreservation when pre-freeze and post-thaw culture was used (160).

Cryopreservation has also been successful with adult rat islets. Cooling with a 2-step procedure (161) gave the same response to glucose challenge as in controls (162) and it was found that cultured islets tolerated the stresses of cooling and thawing better (163,164). Culture after thawing resulted in 87% survival histologically and 75% functionally (165,166), and islets equilibrated stepwise in 2M DMSO, cooled slowly at (0.25°C/min), warmed at 7.5°C/min and cultured for 24 hours after removal of the hyperosmotic protectant, had high survival rate during perifusion (167). Fast cooling as suggested by Bank (153) has also been successful resulting in a net insulin release into culture medium on glucose challenge of islets cooled at 75°C/min in 1M DMSO and thawed at 3.5°C/min that was 75% of that released by nonfrozen controls. Varying protocols have also been used to cryopreserve rat islets which have successfully reversed hyperglycemia following transplantation into diabetic rats. Bretzel et al cooled islets at 2°C/min to -35°C, at

7°C/min to -100°C and then abruptly to -196°C (168,169). Similar success has been reported by Rajotte and co-workers when islets were cooled at 0.25°C/min to -75°C and thawed at 7.5°C/min from -196°C (152). They also found that faster cooling rates resulted in survival of fewer islets and continuation of the diabetic state in all recipients. Subsequent work has led Rajotte et al to modify their cryopreservation protocol so that islets are cooled at 0.25°C/min to -40°C and rapidly thawed at 150°C/min (170). They showed that only 3000 islets were needed to render diabetic rats normoglycemic following slow freezing to -40 and rapid thawing as opposed to 5000 islets when using their previous protocol.

It has also been demonstrated that transplanted cryopreserved pancreatic fragments can normalize carbohydrate metabolism in pancreatectomized dogs and pigs (170-176). The importance of equilibration with 2M DMSO of a temperature of 25°C rather than 0°C for the successful reversal of diabetes by transplantation of canine islets has been documented (171). Rajotte and co-workers have further modified their protocol for cryopreserving canine islets and presently cool in 2M DMSO slowly at a rate of 0.25°C/min to -40°C, plunge to -196°C in liquid nitrogen and thaw rapidly at 150°C/min (173).

The superiority of slow cooling and rapid thawing for the successful autotransplantation of cryopreserved canine microfragments has been demonstrated by other investigators as well (175). Alderson et al successfully produced euglycemia in 3 out of 7 dogs autotransplanted with cryopreserved canine fragments cooled slowly (0.5°C/min) to -50°C prior to immersion in liquid nitrogen and thawed rapidly (80°C/min).

When islets were cooled rapidly (5°C) all 5 dogs autotransplanted remained hyperglycemic.

Recent work has suggested a second and potentially important use for cryopreservation. There is evidence that cryopreservation may purify pancreatic islet cell preparations (89,171). It has been established that varying cell types and multicellular structures have different optimal cryopreservation protocols, particularly cooling and thawing rates (177). This reflects the difference in size, surface to ratio, membrane permeability and tolerance of the cells to osmotic shock and ice crystal formation in these single and multicellular systems (177). These concepts provide a theoretical mechanism whereby cryopreservation protocols optimized for pancreatic islet cells might result in selective destruction of contaminating exocrine tissue. Hinshaw et al have noted the sensitivity of exocrine cells to low temperatures (89). Rajotte et al (171) and Bank (178) have documented evidence of selective destruction of exocrine tissue following cryopreservation. The selective destruction of nonendocrine tissue by cryopreservation may have significant immunological implications as well. Should highly antigenic cells such as passenger leukocytes and dendritic cells be subject to destruction by freezing considerable advantage would be attained in islet allotransplantation (179).

REFERENCES

1. Cahill GF, Arky RA. Diabetes. In: Scientific American Medicine (Rubenstein E, Federman D, eds.). Scientific American Inc. 9(6):1-20, 1983.

2. Foster DW. Diabetes mellitus. In: Harison's Principles of Internal Medicine (Petersdorf RC, Adams RD, Braunwald E, et al, eds.). McGraw-Hill 671-679, 1983.

3. Classification and diagnosis of diabetes mellitus and other categories of glucose intolerance. National Diabetes Data Group. Diabetes 28:1039, 1979.

4. Albin J, Rifkin H. Etiologies of diabetes mellitus. Med Clin North Am 66(6):1209-1225, 1984.

5. Yoon J-W, Austin M, Onoder T, et al. Virus induced diabetes mellitus: isolation of a virus from the pancreas of a child with diabetic ketoacidosis. N Engl J Med 300:1173, 1979.

6. Rossini AA. Why control blood glucose levels. Arch Surg 3:229-233, 1976.

7. Andreani D, Keen H, Squadrito G. Highlights from the international symposium - Diabetes and its Late Complications: New Prospects. Diabetologia 23:284-285, 1982.

8. Bradley WE. Aspects of diabetic autonomic neuropathy. Ann Intern Med 92(suppl 2):293-296, 1980.

9. Tchobroutsky G. Relation of diabetic control to the development of microvascular complications. Diabetologia 15:143-152, 1978.

10. Pirart J. Diabetes mellitus and its degenerative complications: a prospective study of 4400 patients observed between 1947 and 1973. Diabetes Care 1(3):168-188, 1978.

11. Steno Study Group. Effect of 6 months of strict metabolic control on eye and kidney function in insulin-dependent diabetics with background retinopathy. Lancet (Jan 16):121-123, 1982.

12. Eschwege E, Job E, Guyot-Argenton C, Aubry JP, Tchobroutsky G. Delayed progression of diabetic retinopathy by divided insulin administration. A further follow-up. Diabetologia 16:13-15, 1979.

13. Miki E, Fukuda M, Kuzuyu T, et al. Relation of the course of retinopathy to control of diabetes, age and therapeutic agents in diabetic Japanese patients. Diabetes 18(2):773-780, 1968.

14. Engerman R, Bloodworth JMB, Nelson S. Relationship of microvascular disease in diabetes to metabolic control. Diabetes 26(8):760-769, 1977.

15. Lee CS, Mauer MS, Brown DM, et al. Renal transplantation in diabetes mellitus in rats. J Exp Med 139:793-800, 1974.

16. Gray BN, Watkins B. Prevention of vascular complications of diabetes by pancreatic islet cell transplantation. Arch Surg 3:254-257, 1976.

17. Wellman KF, Volk BW. Historical Review. In: The Diabetic Pancreas (Volk BW, Wellman KF, eds.). Plenum Press 1-14, 1977.

18. Unger RH. Benefits and risks of meticulous control of diabetes. Med Clin North Am 66(6):1317-1324, 1982.

19. Tamborlane WV, Press MC. Insulin infusion pump treatment of type 1 diabetes. Ped Clin North Am 31(3):721-734, 1984.

20. Raskin P. Treatment of insulin dependent diabetes mellitus with portable insulin devices. Med Clin North Am 66(6):1269-1282, 1982.

21. Reeves ML, Seigler DE, Ryan EA, Skyler JS. Glycemic control in insulin-dependent diabetes mellitus. Am J of Med 72:673-680, 1982.

22. Tamborlane WV, Sherwin RS. Diabetes control and complications: new strategies and insights. J Pediatr 102:805, 1983.

23. Brown J, Mullen Y, Clark W, et al. Importance of hepatic circulation for insulin action in streptozotocin-diabetic rats transplanted with fetal pancreas. J Clin Invest 64:1688-1694, 1979.

24. Sutherland DER. Pancreas and pancreatic islet cell transplantation. 1. Experimental studies. Diabetologia 20:161-183, 1981.

25. Reemsta K, Weber CJ. Pancreas and pancreatic islet cell transplantation. In: Davis and Christopher Textbook of Surgery (Sabiston DC, ed). WB Saunders Co. 537-542, 1981.

26. Hoffman L, Mandel TE, et al. A comparison between islet transplantation and parenteral insulin in the control of diabetes and prevention of renal complications in mice. Metabolism 32(5):451-456, 1983.

27. Federlin DF, Bretzel BG. The effects of islet transplantation on complications in experimental diabetes of the rat. World J Surg 8:169-178, 1984.

28. Weil R, et al. Pancreatic transplantation in diabetic rats: renal function, morphology, ultrastructure and immunohistology. Surgery 78(2):142-148, 1975.

29. Mauer MS, et al. Pancreatic islet transplantation effects on the glomerular lesions of experimental diabetes in rats. Diabetes 23(9):748-753, 1974.

30. Mauer MS, et al. Studies of the rate of regression of the glomerular lesions in diabetic rats treated with pancreatic islet transplantation. Diabetes 24(3):280-285, 1975.

31. Krupin T, et al. Ocular fluorophotometry in experimental diabetes mellitus: the effect of pancreatic islet isografts. Invest Opthalmol and Visual Sci 18:1185, 1979.

32. Nelson J, Lacy P, Hirschberg G. Megacolon and autonomic neuropathy in diabetic rats. J Neuropath Exp Neurol 35:335, 1976.

33. von Mering J, Minkowski O. Diabetes mellitus nach pankreasextirpation. Arch Exp Path Pharmacologie 26:371, 1889.

34 Wellman KF, Volk BW. Historical review. In: The Diabetic Pancreas (Volk BW, Wellman KF, eds.). Plenum Press 1-14, 1977.

35. Downing R. Historical review of pancreatic islet transplantation. World J Surg 8:137-142, 1984.

36. van Beek C, Leonid V. Sobolev 1876-1919. Diabetes, 7(3):146-248, 1979.

37. Sutherland DER. Pancreas and islet transplantation. 2. Clinical Trials. Diabetologia 20:435-450, 1981.

38. Groth CG. Clinical pancreatic transplantation. Trans Proc 17(11):302-306, 1984.

39. Barker CF, Naji A, Perloff LJ, Dafoe DC, Bartlett S. Invited commentary. An overview of pancreas transplantation-biological aspects. Surgery 92(2):133-137, 1982.

40. Gleidman ML, Gold M, Whittaker J, Rifkin H, Soberman R, Freed S, Tellis V, Veith F. Pancreatic duct to ureter anastomosis for exocrine drainage in pancreatic transplantation. Am J Surg 125:245-252, 1973.

41. Groth C, Tyden G, Lundgren G, Wilczek H, Klintmalm G, Ost L, Gunnarsson R, Ostman J. Segmental pancreatic transplantation with enteric exocrine diversion. World J Surg 8:257-261, 1984.

42. Kyriakides GK, Rabinovitch A, Mintz D, Olson L, Rappaport F, Miller J. Long-term study of vascularized free-draining intraperitoneal pancreatic segmental allografts in beagle dogs. J Clin Invest 67:292-303, 1981.

43. Dubernard J, Traeger J, Bosi E, Gelet A, Yafi S, Devonec M, Piatti PM, Chiesa R, Martin X, Monein-Long B, Jouraine J, Pozza G. Transplantation for the treatment of insulin-dependent diabetes: clinical experience with polymer obstructed pancreatic grafts using neoprene. World J Surg 8:262-266, 1984.

44. Sutherland DER, Moudry KC. Pancreas transplant registry report. Trans Proc 19(4):5, 1987.

45. Groth CG. Clinical pancreatic transplantation. Trans Proc 17(1):302, 1985.

46. Lacy P. Pancreatic transplantation as a means of insulin delivery. Diabetes Care 5(suppl 1):93, 1982.

47. Scharp D. Transplantation of pancreatic islet cells - introduction. World J Surg 8:136-139, 1984.

48. Lacy P. Experimental immunoalteration. World J Surg 8:198-203, 1984.

49. Rajotte RV, Warnock GL, Kneteman NM. Cryopreservation of insulin-producing tissue in rats and dogs. World J Surg 8:179-186, 1984.

50. Sutherland DER, Matas AJ, Najarian JS. Pancreatic islet cell transplantation. Surg Clin North Am 58(2):365-382, 1978.

51. Moskalewski S. Isolation and culture of the islets of Langerhans of the guinea pig. General and Comparative Endocrinology 5:342-353, 1965.

52. Lacy PE, Kostianowsky M. Method for isolation of intact islets of Langerhans from the rat pancreas. Diabetes 16(1):35-39, 1967.

53. Sorenson RL. Isolation of an insulin secretion granule rich from rat islets. Anat Rec 160:498, 1968.

54. Finke EH, Lacy PE, Ono J. Use of reflected green light for specific identification of islets in vitro after collagenase isolation. Diabetes 28:612-613, 1979.

55. Lacy PE, Walker MM, Finke CJ. Perifusion of isolated rat islets in vitro. Diabetes 21:987, 1972.

56. Younoszai R, Sorenzon RL, Lindall AW. Hmeotransplantation of isolated pancreatic islets. Diabetes 19(suppl 1):406, 1970.

57. Ballinger WF, Lacy PE. Transplantation of intact pancreatic islets in rats. Surgery 2:175-186, 1972.

58. Reckard C, Barker C. Transplantation of isolated pancreatic islets across strong and weak histocompatibility barriers. Trans Proc 5(1):761-763, 1973.

59. Kemp CB, Knight MJ, Scharp DW, Ballinger WF, Lacy PE. Effect of transplantation site on the results of pancreatic islet isografts in diabetic rats. Diabetologia 9:486-491, 1973.

60. Tze WJ, Tai J. Successful intracerebral allotransplantation of pancreatic endocrine cells in spontaneously diabetic BB rates without immunosuppression. Metabolism 33(9):785-789, 1984.

61. Leonard RJ, Lazarow A, Hegre O. Pancreatic islet transplantation in the rat. Diabetes 22:413-418, 1973.

62. Mullen Y, Clark WR, Kemp J, Molnar JG, Brown J. Reversal of experimental diabetes by foetal rat pancreas. II. Critical procedures for transplantation. Trans Proc 9(1):329-332, 1977.

63. Brown J, Clark WR, Molnar JG, Mullen YS. Foetal pancreas transplantation for reversal of streptozotocin induced diabetes in rats. Diabetes 25:56-65, 1976.

64. McEvoy R, Hegre O. Syngeneic transplantation of foetal rat pancreas. III. Effect of insulin treatment on the growth and differentiation of pancreatic implants after reversal of diabetes. Diabetes 38:141-146 1979.

65. Zeigler M, Reckard C, Barker C. Long-term metabolic and immunologic considerations in transplantation of pancreatic islets. J Surg Res 16:575-581, 1974.

66. Scharp D. Isolation and transplantation of islet tissue. World J Surg 8:143-151, 1984.

67. Mirkovitch V, Campiche M. Intrasplenic autotransplantation of canine pancreatic tissue: maintenance of normoglycemia after total pancreatectomy. Eur Surg Res 9:173-190, 1977.

68. Kretschmer GJ, Sutherland DER, Matas AF, Steffes MW, Najarian JS. The dispersed pancreas: transplantation without islet purification in totally pancreatectomized dogs. Diabetologia 13:495-502, 1977.

69. Warnock GL, Rajotte RV, Procyshyn AW. Normoglycemia after reflux of islet containing fragments into the splenic vascular bed in dogs. Diabetes 32(5):452-458, 1983.

70. Kolb E, Ruckert R, Largardier F. Intraportal and intrasplenic autotransplantation of pancreatic islets in the dog. Eur Surg Res 9:419-426, 1977.

71. Kretschmer GJ, Sutherland DER, Matas AJ, Payne WD, Najarian JS. Autotransplantation of pancreatic fragments to the portal vein and spleen of totally pancreatectomized dogs. Ann Surg 187(1):79-86, 1978.

72. Mehigan DG, Bell NR, Zuidema GD, Eggleston JC, Cameron JL. Disseminated intravascular coagulation and portal hypertension following pancreatic islet autotransplantation. Ann Surg 191(3):287-293, 1980.

73. Lorenz D, Lippert H, Tietz W, Worm V, Hann NJ, Dorn A, Koch G, Ziegler M, Rosenbaum KD. Transplantation of isolated islets of Langerhans in diabetic dogs. I. Results after allogenic intraportal islet transplantation. J Surg Res 27:181-192, 1979.

74. Mieny CJ, Smit JA. Autotransplantation of pancreatic tissue in totally pancreatectomized baboons. S Afr J Surg 16:19-21, 1978.

75. Scharp DW, Murphy JJ, Newton WT, Ballinger WF, Lacy P. Transplantation of islets of Langerhans in diabetic rhesus monkeys. Surgery 77(1):100-105, 1975.

76. Kiviluoto T. Transplantation of pancreatic microfragments in totally pancreatectomized pigs. Eur Surg Res 17:119-127, 1985.

77. Toledo-Peyera LH, Bandlein KO, Gordon DA, MacKenzie GH, Reyman TA. Renal subcapsular islet cell transplantation. Diabetes 33:910-914, 1984.

78. Sutherland DER. Pancreas and islet transplant registry data. World J Surgery 8:270-275, 1984.

79. Sutherland DER, Kendal KD. Clinical pancreas and islet transplant registry report. Trans Proc 17(1):307, 1985.

80. Alderson D, Farndon JR. The metabolic effects of islet transplantation in the diabetic dog. Trans Proc 16(3):831-833, 1984.

81. Scharp DW, Downing R, Merrel RC, Greider M. Isolating the elusive islet. Diabetes 29(suppl 1):19-30, 1980.

82. Matas AJ, Sutherland DER, Steffes MW, Najarian JS. Islet transplantation using neonatal rat pancreas: quantitative studies. J Surg Res 20:143-147, 1976.

83. Horaguchi A, Merrel RC. Preparation of viable islet cells from dogs by a new method. Diabetes 30:455-458, 1981.

84. Payne WD, sutherland DER, Matas AJ, Gorecki P, Najarian JS. DL-ethionine treatment of adult pancreatic donors. Amelioration of diabetes in multiple recipients with tissue from a single donor. Ann Surg 189(2):148-256, 1979.

85. Vrbova H, Theodorov NA, Tyhurst M, Howell SL. Transplantation of islets of Langerhans from pilocarpine-pretreated rats. A method of enhancing islet yield. Transplantation 28(5):433-435, 1979.

86. Downing R, Scharp DW, Ballinger WF. An improved technique for isolation and identification of mammalian islets of Langerhans. Transplantation 29(29):79-83, 1980.

87. Gray DWR, McShane P, Morris PJ. A method for isolation of islets of Langerhans from the human pancreas. Trans Proc 17(1):381-382, 1985.

88. Lacy PE, Lacy ET, Finke EH, Yasunami Y. An improved method for the isolation of islets from the beef pancreas. Diabetes 31(suppl 4):109-111, 1982.

89. Hinshaw DB, Jolley WB, Hinshaw DB, Kaiser JE, Hinshaw K. Islet autotransplantation after pancreatectomy for pancreatitis with a new method of islet preparation. Am J Surg 142:118-122, 1981.

90. Scharp DW. Clinical feasibility of islet transplantation. Trans Proc 16(3):820-825, 1984.

91. Lazarow A, Wells LJ, Carpenter AM, Hegre O, Leonard RJ, McEvoy RC. Islet differentiation, organ culture and transplantation. Diabetes 22(1):877-912, 1973.

92. Matas AJ, Sutherland DER, Steffes MW, Najarian JS. Short-term culture of adult pancreatic fragments for purification and transplantation of islets of Langerhans. Surgery 80(2):183-191, 1976.

93. Matas AJ, Sutherland DER, Kretschmer G, Steffes MW, Najarian JS. Pancreatic tissue culture: depletion of exocrine enzymes and purification of islets for transplantation. Trans Proc 9(1):337-339, 1977.

94. Lundgren G, Andersson A, Borg H, Buschard K, Groth CG, Gunnarrson R, Hellerstrom C, Ostman J. Structural and functional integrity of isolated human islets of Langerhans maintained in tissue culture for 1-3 weeks. Trans Proc 9(1):237-240, 1977.

95. Weber CJ, Hardy MA, Lerner RL, Reemsta K. Tissue culture isolation and preservation of human cadaveric pancreatic islets. Surgery 81(3):270-273, 1977.

96. Lindhall PE. Principle of a counter-streaming centrifuge for the separation of particles of different sizes. Nature 164:648-649, 1948.

97. Nason RW, Rajotte RV, Procyshyn AW, Pederson JE. Purification of canine pancreatic islet cell grafts with radiation. Trans Proc 18(1):174-181, 1986.

98. Noel J, Rabinovitch A, Olsen L, Kyriakides G, Miller J, Mintz DH. A method for large scale, high yield isolation of canine pancreatic islets of Langerhans. Metabolism 31(2):184-187, 1982.

99. Alejandro R, Cutfield R, Shienvold FL, Latif Z, Mintz DH. Successful long-term survival of pancreatic islet allografts in spontaneous or pancreatectomy-induced diabetes in dogs. Cyclosporine-induced immune unresponsiveness. Diabetes 34:825-828, 1985.

100. Warnock GL, Rajotte RV. Critical mass of purified islets that induce normoglycemia after implantation into dogs. Diabetes 37:467-470, 1988.

101. Rechard CR, Ziegler MM, Barker CF. Physiologic and immunological consequences of transplanting isolated pancreatic islets. Surgery 74:91-99, 1973.

102. Perloff LJ, Naji A, Barker CF. Islet sensitivity to humoral antibody. Surg Forum 32:390-391, 1981.

103. Finch DRA, Morris PJ. The effect of increasing islet numbers on survival of pancreatic islet allografts in immunosuppressed diabetic rats. Transplantation 23(1):104-106, 1977.

104. Lacy PE, Davie JM, Finke EH. Prolongation of islet allograft survival following in vitro culture (24°C) and a single inject of ALS. Science 204:312-313, 1979.

105. Lacy PE. Experimental immunoalteration. World J Surg 8:198-203, 1984.

106. Lafferty K, Cooley M, Woolnough J, Walker K. Thyroid allograft immunogenicity is reduced after a period in organ culture. Science 188:259-261, 1975.

107. Lafferty KJ, Prowse SJ. Theory and practice of immunoregulation by tissue treatment prior to transplantation. World J Surg 8:187-197, 1984.

108. Lacy PE, Davie J, Finke E. Induction of rejection of successful allografts of rat islets by donor peritoneal exudate cells. Transplantation 28(5):415-420, 1979.

109. Parr E. The absence of H_2 antigens from mouse pancreatic B cells demonstrated by immunoferritin labelling. J Exp Med 150:1-9, 1979.

110. Faustman D, Hauptfeld V, Davie J, Lacy P, Shreffler D. Murine pancreatic β-cells express H-2K and H-2D but not Ia antigens. J Exp Med 151:1563-1568, 1980.

111. Shienvold FL, Alejandro R, Mintz DH. Identification of Ia bearing cells in rat, dog pig and human islets of Langerhans. Transplantation 41(3):364-372, 1986.

112. Hart DNJ, Newton MR, Reece-Smith H, Fabre JW, Morris PJ. Major histocompatibility complex antigens in the rat pancreas, isolated pancreatic islets, thyroid and adrenal. Transplantation 36(4):431-435, 1983.

113. Ulrichs K, Muller-Ruchholtz. MHC class II antigen expression on the various cells of normal and activated isolated pancreatic islets. Diagnostic Immunology 3:47-55, 1985.

114. Bowen K, Andrus L, Lafferty K. Successful allotransplantation of mouse pancreatic islets to nonimmunosuppressed recipients. Diabetes 29:98-103, 1980.

115. Lacy PE, Finke E, Janney C, Davie J. Prolongation of islet xenograft survival by in vitro culture of rat megaislets in 95% O_2. Transplantation 33(6):588-592, 1982.

116. Faustman DL, Steinman RN, Gebel HM, Hauptfeld V, Davie JM, Lacy PE. Prevention of mouse islet allograft rejection by elimination of intraislet dendritic cells. Trans Proc 17(1):420-422, 1985.

117. Hardy MA, Lau HT, Reemsta K. Prolongation of rat islet allografts with the use of ultraviolet irradiation, without immunosuppression. Trans Proc 16(3):865-869, 1984.

118. Faustman D, Lacy P, Davie J, Hauptfeld V. Prevention of allograft rejection by immunization with donor blood depleted of Ia-bearing cells. Science 217:157-158, 1982.

119. Lau H, Reemsta K, Hardy MA. Pancreatic islet allograft prolongation by donor-specific blood transfusions treated with ultraviolet radiation. Science 221:754-755, 1983.

120. Gray DWR, Reece-Smith H, Fairbrother B, et al. Survival of isolated pancreatic islets in rats rendered immunologically unresponsive to renal allografts. Trans Proc 17(3):870-871, 1984.

121. Scharp DW, NAson RS, Sparks RE. Islet immunoisolation and the use of hybrid artificial organs to prevent islet tissue rejection. World J Surg 8:221-229, 1984.

122. van Brunt J. Vinotech makes a bioartifical pancreas. Biotechnology 3:853-854, 1985.

123. Nash JR, Bell PR. Effect of macrophage suppression on the survival of islet allografts. Trans Proc 11:986-988, 1979.

124. Kolb E, Urfer K, Largradier F. Early rejection of allotransplanted pancreatic islets in the dog. Trans Proc 11:543-548, 1979.

125. Kretchmer GJ, Sutherland DER, Matas AJ, Najarian JS. Preliminary experience with allotransplantation of pancreatic fragments to the spleen of totally pancreatectomized dogs. Trans Proc 11:537-542, 1979.

126. Frangipane LG, Barker CF, Silvers WK. Importance of weak histocompatibility factors in survival of pancreatic islet transplants. Surgical Forum 28:294-296, 1977.

127. Beyer MM, Friedman EA. Histocompatibility-dependent long-term islet of Langerhans survival induced by antithymocyte globulin. Trans Proc 11:1436-1439, 1979.

128. Barker CF, Fragnipane LG, Silvers WK. Islet transplantation genetically determined diabetes. Annals of Surgery 186:40[illegible]10, 1977.

129. Lorenz D, Lippert H, Panzig E, et al. Transplantation of isolated islets of Langerhans in diabetic dogs III. Donor selection by mixed lymphocyte reaction and immunosuppressive treatment. J Surg Res 79:205-213, 1979.

130. Gray DWR, Morris PJ. Cyclosporine and pancreas transplantation. World J Surg 8:230-235, 1984.

131. Garvey JF, McShane P, Poole MD, Millard PR, Morris PJ. The effect of cyclosporine A on experimental pancreas allografts in the rat. Trans Proc 12:266, 1980.

132. Rynasiewicz J, Sutherland DE, Kawahara K, Gorecki P, Najarian JS. Cyclosporine A prolongation of segmental pancreatic and islet allograft function in rats. Trans Proc 12:270, 1980.

133. McMaster P, Procyshyn A, Calne RY, Valdes R, Rolles K, Smith DJ. Prolongation of canine pancreas allograft survival with cyclosporine A. Preliminary report. Br Med J 1:444, 1980.

134. Rynasiewicz J, Sutherland DE, Kawahara K, Gorecki P, Najarian JS. Cyclosporine A prolongation of segmental pancreatic and islet allograft function in rats. Trans Proc 12:270, 1980.

135. Vialettes B, Simon MC, Lassmann V, Vague P. Prolonged survival of allotransplanted islets of Langerhans after cyclosporine A treatment in rats. Transplantation 28:435, 1979.

136. Bell PR, Wood RF, Peters M, Nash JR. Comparison of various methods of chemical immunosuppression in islet cell transplantation. Trans Proc 12:291, 1980.

137. Kneteman NM, Rajotte RV, Procyshyn AW. Canine pancreatic fragment allotransplantation with cyclosporine A. J Surg Res 39:285-293, 1985.

138. Najarian J, Goetz FC. Twin to twin pancreas transplantation: reversal and re-enactment of the pathogenesis of type 1 diabetes. Trans Assoc Am Physicians 97:80-87, 1984.

139. Naji A, Silvers WK, Bartlett ST, Francfort J, Barker C. Immunologic factors in pathogenesis and treatment of human and animal diabetes. World J Surg 8:214-220, 1984.

140. Albisser AM, Nomura M, Greenberg GR, McPhedran NT. Metabolic control in diabetic dogs healed with pancreatic autotransplants and insulin pumps. Diabetes 35:97-100, 1986.

141. Yasunami Y, Lacy PE, Finke EH. A new site for islet transplantation - a peritoneal O mental pouch. Transplantation 36(2):181-182, 1983.

142. Wabh TJ, Eggleston JC, Cameron JL. Portal hypertension, hepatic infarction and liver failure complicating pancreatic islet autotransplantation. Surgery 91:485-487, 1982.

143. Memsic L, Busuttil RW, Traverso LW. Bleeding esophageal varices and portal vein thrombosis after pancreatic mixed cell autotransplantation. Surgery 95(2):238-242, 1984.

144. Traverso WL, Gomez RG. Hemodynamic measurements after administration of aprotinin and/or heparin during pancreatic cell autotransplantation in dog, pig and monkey. Ann Surg 195:479-485, 1982.

145. Torres LE, Traverso WL, Yound ZS. Intraoperative hemodynamic changes in patients undergoing mixed-cell intraportal autotransplantation of pancreatic tissue. Anaesthesiology 53:427-429, 1980.

146. Hesse UJ, Sutherland DER. Comparison of splenic and renal subcapsular islet autografting in dogs. Transplantation 41(2):271-274, 1986.

147. Knight MJ, Scharp DW, Kemp CB, Nunnelly SB, Ballinger WF, Lacy PE, Effects of cold storage on the function of isolated pancreatic islets. Cryobiology 10:89-90, 1973.

148. Frankel BJ, Gylfe E, Hellman B, Idalh LA: Maintenance of insulin release from pancreatic islets stored in the cold for up to 5 weeks. J Clin Invest 57:47-52, 1976.

149. Schulak JA, Stuart FP, Reckard CR. Physiologic aspects of intrasplenic autotransplantation of pancreatic fragments in the dog after 24 hours of cold storage. J Surg Res 24:125-131, 1978.

150. Anderson A, Buschard F. Culture of isolated pancreatic islets: its application for transplantation purposes. Trans Am Soc Artif Intern Organs 23:342-343, 1977.

151. Nakagawara G, Yamasaki G, Kimura S, Kajima Y, Muzazaki I. Insulin releasing activity and successful transplantation of pancreatic islets preserved by tissue culture. Surgery 83:188-193, 1978.

152. Rajotte RV, Scharp DW, Downining R, Preston R, Molnar GD, Ballinger WF, Greider MH. Pancreatic islet banking: the transplantation of frozen-thawed rat islets transported between centers. Cryobiology 18:357, 1981.

153. Bank HL, Davis RF, Emerson D. Cryogenic preservaiton of isolated rat islets of Langerhans: effect of cooling and warming rates. Diabetologia 16:195, 1979.

154. Federlin K, Bretzel RG, eds. Islet isolation, culture and cryopreservation. Stuttgart, New York, Thieme-Stratton, Inc. 124-165, 1981.

155. Bank HL, ed. Symposium on cryobiology of isolated islets of Langerhans. Cryobiology 20:119-245, 1983.

156. Mazur P, Kemp JA, Miller RH. Survival of fetal pancreases frozen to -78° and -196°C. Proc Natl Acad Sci USA 73:4105, 1976.

157. Mazur P, Rajotte RV. Permeability of the 17-day fetal rat pancreas to glycerol and dimethyl sulfoxide. Cryobiology 18:1, 1981.

158. Kemp JA, Mullen Y, Weissman H, Heininger D, Brown J, Clark WR. Reversal of diabetes in rats using fetal pancreases stored at -196°C. Transplantation 26:260, 1978.

159. Payne WD, Sutherland DER, Matas AJ, Najarian JS. Cryopreservation of neonatal rat islet tissue. Surg Forum 29:347, 1978.

160. Brown J, Kemp JA, Hurt S, Clark WR. Cryopreservation of human fetal pancreas. Diabetes 29(suppl 1):70, 1980.

161, McGann LE, Farrant J. Survival of tissue culture cells frozen by a two-step procedure to -196°C. I. Holding temperature and time. Cryobiology 13:261, 1976.

162. Bank HL, Reichard L. Cryogenic presérvation of isolated islets of Langerhans: two-step cooling. Cryobiology 18:489, 1981.

163. Sandler S, Nilsson B, Borg LAH, Swenne I, Petersson B, Hellerstrom C, Andersson A. Structure and function of cryopreserved mouse pancreatic islets. In: Islet Isolation, Culture and Cryopreservation (Federlin K, Bretzel RG, eds.) Stuttgart, New York, Thieme-Stratton, Inc. 138-151, 1981.

164. Sandler S, Andersson A. The significance of culture for successful cryopreservation of isolated pancreatic islets of Langerhans. Cryobiology 21:503-510, 1984.

165. Furguson J, Allsopp RH, Taylor RMR, Johnston IDA. Isolation and long-term preservation of pancreatic islets from mouse, rat and guinea pig. Diabetologia 12:115, 1976.

166. Ferguson J, Allsopp RH, Taylor RMR, Johnston IDA. Isolation and preservation of islets from the mouse, rat, guinea pig and human pancreas. Br J Surg 63:767, 1976.

167. Rajotte RV, Stewart HL, Voss WAG, Shnitka TK, Dossetor JB. Viability studies of frozen-thawed rat islets of Langerhans. Cryobiology 14:116, 1977.

168. Bretzel RG, Schneider J, Zekorn T, Federlin K. Cryopreservation of rat, porcine and human pancreatic islets for transplantation. in:

Islet Isolation, Culture and Cryopreservation (Federlin K, Bretzel RG, eds.) Stuttgart, New York, Thieme-Stratton, Inc. 152-160, 1981.

169. Bretzel RG, Schneider J, Dobroschke J, SchwemmLe K, Pfeiffer EF, Federlin K. Islet transplantation in experimental diabetes of the rat. VII. Cryopreservation of rat and human islets. Preliminary results. Horm Metab Res 12:274, 1980.

170. Rajotte RV, Warnock GL, Kneteman NM. Cryopreservation of insulin producing tissue in rats and dogs. World J Surg 8:179-186, 1984.

171. Rajotte RV, Warnock GL, Bruch LC, Procyshyn AW. Transplantation of cryopreserved and fresh rat islets and canine pancreatic fragments comparison of cryopreservation protocols. Cryobiology 20:169 1983.

172. Kneteman NM, Rajotte RV, Warnock GL. Long-term Normoglycemia in Pancreatectomized Dogs Transplanted with Frozen/Thawed Pancreatic Islets. Cryobiology 23:214-221,1986.

173. Rajotte RV, Warnock GL, Kneteman NM. Autotransplantation of fresh and cryopreserved canine islets. Diab Res Clin Practice. XII Congress of the International Diabetes Federation (1985) Suppl 1, no. 1197, S460 (Madrid, Spain 23-28 September 1985).

174. Wise MH, Gordon C, Johnson RWG. Intraportal autotransplantation of cryopreserved porcine islets of Langerhans. Cryobiology 22:359-366, 1985.

175. Alderson D, Walsh TN, Farndon JR. Islet cell transplantation in diabetic dogs: studies of graft function and storage. Br J Surg 71:756-760, 1984.

176. Walsh TN, Alderson D, Farndon JR. Successful autotransplantation of cryopreserved dispersed pancreatic grafts in dogs. Transplantation 38(5):546-548, 1984.

177. Rajotte RV, Mazur P. Survival of frozen-thawed fetal rat pancreases as functions of the permeation of dimethyl sulfoxide and glycerol, warming rate and fetal age. Cryobiology 18:17, 1981.

178. Bank HL. A high yield method for isolating rat islets of Langerhans using differential sensitivity to freezing. Cryobiology 20:237-244, 1983.

179. Coulombe MG, Warnock GL, Rajotte RV. Prolongation of islet xenograft survival by cryopreservation. Diabetes 36(9):1086, 1987.

II

CRYOPRESERVATION PURIFIES CANINE PANCREATIC MICROFRAGMENTS[1]

Experimental islet cell transplantation is very successful in syngeneic strains of rodents where purified islets from multiple donors can normalize serum glucose and reverse microvascular complications in diabetic recipients (1-3). The techniques developed in the rodent model have proved ineffective for isolating sufficient islets from the compact pancreas of large mammals and humans. A partial solution to this problem, validated in the canine model, has been to eliminate the steps of purification which reduce islet yields, and collect islet-containing pancreatic fragments (4). However, the presence of contaminating exocrine cells in these grafts has resulted in unacceptable complications (5-7), thus precluding effective clinical trials (8).

To promote clinical trials, new approaches are needed to store sufficient islets to treat individual recipients and purify islet grafts. Cryopreservation is an effective method for storage of islet grafts (9,10,11). Furthermore, cryopreservation may destroy contaminating exocrine cells, an observation which concurs with the reputed sensitivity of exocrine tissue to cold (11,12). The ability to remove harmful exocrine tissue from dispersed pancreatic tissue would obviate portal hypertension. The present study examines the effect of cryopreservation on pancreatic microfragment purity. Function of the cryopreserved autograft was compared with that of fresh tissue following intraportal embolization in a large-mammal transplant model.

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MATERIALS AND METHODS

Experimental Design

Intravenous glucose tolerance (ivGTT) was tested in 12 dogs, then total pancreatectomy was performed. Tissue was removed from fresh pancreas and freshly prepared and cryopreserved grafts for determination of insulin and amylase content. Six dogs received cryopreserved tissue by portal vein embolization while an equal number of control animals received fresh tissue. Portal pressure was measured before and after embolization to the liver. Autograft function was assessed by fasting plasma glucose and insulin determinations daily for the first week and weekly thereafter. A second ivGTT was performed 1 month following transplantation. Animals were sacrificed when they lost 25% of preoperative weight in the face of hyperglycemia. Samples of freshly prepared and cryopreserved grafts were fixed in 10% formalin for staining with Gomori's Aldehyde Fuchsin.

Animals and Care

Twelve mongrel dogs of both sexes weighing 17-26.3 kg were studied. Surgical procedures were carried out under general anesthesia with sodium pentobarbital, 30 mg/kg/body weight. The dogs were weighed weekly and permitted unrestricted exercise twice daily. Full diet consisted of 360 g of meat (Dr. Ballard's, Nabisco, Toronto, ON) and 600 g of Burger Bits (Pow R Pac, Swifts, Calgary, AB). On postoperative day 1 hydration was maintained with 1 L of normal saline injected subcutaneously. On postoperative day 2 water was allowed, on day 3 a meat diet was offered and on day 4 full diet resumed. Each meal was supplemented with 8 capsules of Cotazym (Organon Canada Ltd., Montreal, PQ). Prophylactic antibiotics, Derapen-C (Ayerst, Montreal, PQ) 3-4 mL,

were administered at the onset of surgery, and on postoperative day 1. Until cryopreserved grafts were thawed and reimplanted, daily subcutaneous injections of 6-8 units of NPH insulin (Connaught Laboratories, Willowdale, ON) were administered.

Pancreatectomy

The dogs were anesthetized with sodium pentobarbital (30 mg/kg) then pancreatectomy was performed as described in detail by Cobb and Merrel (13). Through an upper midline incision the entire pancreas was mobilized with the 4 major vascular connections preserved. Both branches of the pancreatic duct were cannulated (PE90 polyethylene tubing) in situ. Finally, the blood vessels were clamped, ligated and the gland removed. Immediately the pancreas was weighed and ducts distended by injection of Hank's balanced salt solution (HBSS, Gibco, Chagrin Falls, OH) approximately 60 [illegible]e horizontal limb (body and tail) and 20 mL into the vertic[illegible]ncinate process). The gland was immersed in HBSS at 4°C and [illegible]ed to the laboratory for graft preparation. When transplanting fresh tissue an assistant maintained the dogs under general anesthesia during graft preparation. Cryopreserved grafts were transplanted 24-48 h later at a second laparotomy.

Graft Preparation

The graft was prepared by a method previously reported from this lab by Warnock (14). Briefly, the cannulas were connected to a perfusion apparatus and flushed with chilled (4°C) HBSS at a pressure of 300 mmHg. The perfusate was then changed to a solution of 0.4% collagenase (Type V, 490 U/mg, Sigma Chemicals, St. Louis, MO) in HBSS at 37°C. The flow was in a retrograde direction at a pressure of 300

mmHg through ducts of decreasing size. The gland was suspended on a 60-μm mesh above the reservoir of collagenase from which the enzyme was reperfused. When the gland was mushy or mucoid (average 24 min) digestion was judged complete. The digested gland was bisected and transferred to 2 jars containing 50 mL of enzyme-stop solution consisting of 4°C HBSS supplemented with 2% Trasylol (Miles Pharmaceuticals, Rexdale, ON), penicillin 100 U/mL and streptomycin 100 ug/mL. The tissue was minced for exactly 90 seconds in a mechanical mincer (15). The supernatant fluid was discarded and the preparation resuspended in fresh enzyme-stop solution and transferred to an Ehrlenmeyer flask. Dissociation was accomplished by shaking the flask vigorously in an Evapomix for 10 min at 4°C, followed by filtration through a 400-μm screen. Residual unfiltered tissue was returned to the Ehrlenmeyer flask and passed through the dissociation process again. Finally, the preparation was washed x3 in enzyme-stop solution. The filtrate was centrifuged at 2000 rpm for 20 seconds and supernatant fluid removed. The pellet of islet tissue was cryopreserved (n=6) or resuspended in 30-40 cc of enzyme-stop solution for immediate autotransplantation (n=6). These procedures were performed in a laminar flow hood with the time for graft preparation being approximately 2 h.

Cryopreservation

Freezing and thawing were by methods previously reported (11) with modifications as follows. The tissue pellet was resuspended at 22°C in Medium 199 with 10% fetal calf serum, 2% aprotinin, penicillin 200 U/mL and streptomycin 200 ug/mL (v/v). A volume equal to that of the original tissue pellet of 2M dimethyl sulfoxide (Me_2SO) in the same Medium 199 mixture was then added to the Ehrlenmeyer flask at 25°C.

After 5 min another volume of 2M Me_2SO solution was then added and shaking continued for 25 min. Four volumes of 3M Me_2SO solution were then added (final Me_2SO concentration in islet-bearing solutions was 2M) followed by shaking at 25°C for 15 min.

The tissue suspension (0.5 mL tissue in 4mL suspension) was then aliquoted to 16x125 mm glass KIMAX (Owens, Illinois) test tubes at 4°C. After 10 min the tubes were transferred to an ethanol bath at -7.2°C to supercool for 5 min. The suspension was then nucleated by contacting the tube with stainless steel tubing through which liquid nitrogen (N_2) was pumped by an FTS (FTS Systems Inc.) circulating pump. Fifteen minutes were allowed for release of the latent heat of fusion, then the tubes were transferred to evacuated dewars filled with ethanol and surrounded by liquid N_2 for cooling at 0.25°C/min (16). When the temperature reached -40°C the tubes were plunged into liquid N_2 (-196°C) for storage.

Thawing Procedure

After 24-72 h the tissue was thawed by agitation in a water bath at 37°C (150°C/min). Supernatant was aspirated after centrifugation (1500 rpm) and 2.5 mL of 0.75M sucrose in Medium 199 solution was added to each aliquot to remove the hypertonic Me_2SO from the intracellular space of the pancreatic fragments. After equilibration of the preparation for 30 min at 0°C the sucrose was diluted in 4 concurrent steps of 5 min each by the addition of Medium 199 with 10% FCS, 2% Trasylol, penicillin and streptomycin while shaking at 22°C. The mixture was centrifuged, the pellet washed, spun and resuspended to 30 mL with Medium 199 solution for transplantation.

Transplantation Procedure

Silastic tubing (Dow Corning, Midland, MI, Cat. No. 602-205) was introduced via the gastroduodenal vein remnant and advanced into the portal vein with a 3-way stopcock attached to a manometer to allow monitoring of portal venous pressure. Sodium Heparin (Allen and Hansburgs, Glaxo Canada Ltd., Toronto, ON), 150 USP U/kg was given intravenously. Pancreatic tissue was injected in 6 equal aliquots each infused over 5 min, at intervals of 5 min with a total infusion time of 1 h. The Silastic catheter was flushed with 5 cc of saline containing 1000 USP units of sodium heparin per liter after each aliquot.

Tissue Assays

Samples of pancreas, freshly prepared and cryopreserved grafts were weighed, homogenized and sonicated in 5 mL of chilled 2% acid (H_2SO_4): 80% alcohol. Insulin was extracted over 24 h at 4°C. Samples were neutralized and diluted in saturated sodium bicarbonate (Gibco, Grand Island, New York). Insulin was measured by double-antibody radioimmunoassay (17) using Insulin RIA kits (Pharmacia, Uppsala, Sweden) and human insulin standards (WHO International Lab for Biol. Standards) and amylase with Dri-STAT Amylase-DS reagent (Beckman Instruments, Carlsbad, CA). The ratio of insulin to amylase content was determined as a measurement of purity. The comparison was made under the assumption that tissue insulin and amylase content are proportional to pancreatic β-cell and exocrine tissue mass respectively (18).

Blood Indices

Fasting plasma glucose (PG mg/dL) was measured by the glucose oxidase method using the Beckman Glucose Analyzer 2 (Beckman Instruments, Brea, CA). Immunoreactive insulin (IRI μU/mL) was

determined by double antibody radioimmunoassay using RIA kits (Pharmacia, Uppsala, Sweden). For ivGTT saphenous veins were cannulated, glucose (0.5 g/kg body weight) was injected and blood collected at 0, 1, 5, 10, 15, 30, 60 and 90 min for assays of glucose and insulin. The K value (19), a measure of the decline in glucose level (%/min) was determined from glucose levels at 5, 10, 15 and 30 min.

Analysis of Data

All results are expressed as mean±SEM. The data were analyzed with Student's t-test for paired or unpaired data as applicable. Differences are stated as significant when $p<0.05$ unless indicated otherwise.

RESULTS

Insulin Amylase Ra[illegible] II-1)

Volume, ins[illegible] and [illegible]mylase concentration and insulin/amylase ratios of whole pancreas, freshly processed and cryopreserved grafts are shown. Both insulin and amylase concentration were reduced significantly following the collagenase digestion process. Cryopreservation causes a significant reduction in the amylase concentration while insulin remains unchanged, resulting in a greater than 3-fold increase in the insulin amylase ratio.

Portal Pressure (Figure II-1)

Portal venous pressure increased by 16.4±2.3 cm H_2O (mean±SEM) following intraportal infusion of cryopreserved tissue which was significantly less ($p<0.01$) than that seen following the infusion of freshly isolated tissue (31.2±3.3 cm H_2O).

Survival of Dogs (Table II-2)

Following intraportal embolization, 2 of 6 dogs receiving fresh tissue and 3 of 6 dogs receiving cryopreserved tissue remained normoglycemic. Four dogs engrafted with fresh tissue died before 1 month: 1 due to small bowel infarction; 2 died suddenly within 24 h of transplantation without obvious cause; a fourth died secondary to documented graft failure. Following intraportal engraftment of cryopreserved tissue 1 dog died due to graft failure.

Autograft Function (Figure II-2)

The fasting plasma glucose was equal for 4 weeks following intraportal embolization of freshly prepared or cryopreserved pancreatic fragments. Results from ivGTT preoperatively and 1 month following engraftment are shown (Figure II-3) with only normoglycemic animals being listed at 1 month. K values were significantly reduced ($p<0.01$) in dogs receiving both fresh and cryopreserved tissue intraportally at 1 month.

Histology

Intact islets were observed in fresh grafts and following cryopreservation. Fresh acinar tissue appeared viable, but, following cryopreservation degeneration of exocrine cells was evident with vacuolization of cytoplasm and loss of cellular integrity.

DISCUSSION

Previous studies have suggested that cryopreservation purifies pancreatic microfragments by virtue of the sensitivity of exocrine tissue to cold (11,12). In the present study, purification of canine pancreatic fragments is demonstrated by a greater than 3-fold increase

in the insulin to amylase ratio following cryopreservation. Insulin and amylase concentration were both significantly reduced by the isolation process. In comparison cryopreservation did not affect insulin, but resulted in a significant fall in the concentration of amylase. Histological examination of islet cell preparations showed preservation of intact islets following cryopreservation, however, exocrine tissue no longer appeared viable demonstrating vacuolization and loss of cellular integrity. These findings suggest that cryopreservation purifies pancreatic microfragments at the expense of contaminating exocrine components [illegible] in keeping with the suggested susceptibility of pancreatic [illegible] tissue to injury by the freeze-thaw process (11,12).

It has been established that different cell types have different optimal cooling and thawing rates due to their difference in size, membrane permeability and tolerance to osmotic shock [illegible]. The present results suggest that when this cryopreservation protocol is optimized for pancreatic islet cells, selective destruction of contaminating exocrine cells occurs.

Other methods of purifying canine pancreatic microfragments have been reported. Matas et al demonstrated a greater than 6-fold increase in the insulin/amylase ratio of canine pancreatic fragments cultured for 24 h (15,21). During culture they demonstrated a significant fall in amylase concentration. In contrast to the present study in which the insulin concentration was unchanged following cryopreservation they demonstrated a fall in insulin concentration of over 1/2 that of the whole pancreas during culture. When autografted to diabetic dogs the use of cultured islet tissue resulted in a 50% success rate in normalizing plasma glucose (21). However, partial pancreatectomy and

streptozotocin is a less reliable model of diabetes, as demonstrated by the fact that 1 of their control dogs remained normoglycemic. Nason et al reported the use of high dose radiation to purify canine pancreatic microfragments (22). They demonstrated a 3-fold increase in the insulin/amylase ratio of tissue, following external beam irradiation. Following intrasplenic autoimplants Nason and associates were able to reverse diabetes in 70% of dogs followed for 1 month. The induction of islet cell neoplasia, however, is a possible complication of this technique (23). The use of pseudo-islet formation by Scharp (24) and Ficoll gradient sedimentation (Sigma, St. Louis, MO) by Alejandro et al (25) has allowed the isolation of highly purified preparations of canine islets. Scharp has been able to successfully reverse diabetes in 25% of dogs transplanted to the spleen with pseudo islets derived from single organs. Alejandro and associates have had success transplanting islets purified by Ficoll gradient sedimentation using an allotransplant model, however, insufficient yields of purified islets sometimes necessitated pooling of purified islets from multiple donors.

In the present study, the function of grafts purified by cryopreservation was compared with controls that received fresh tissue. Diabetes was successfully reversed in 3 of 6 dogs following intraportal embolization with cryopreserved tissue compared with 2 of 6 treated with fresh tissue. Results of post-transplant fasting plasma glucose and comparison between K values at 1 month would suggest that the degree of glucose homeostasis achieved with the cryopreserved graft is equal to that of fresh tissue, and that the moderate success rate seen (≈50%) was due to the site of engraftment chosen rather than loss of viability as a result of cryopreservation. The ability to successfully reverse

pancreatectomy-induced diabetes with 100% success using the identical cryopreservation protocol as the present study has been demonstrated when the graft is transplanted to the spleen (10).

Embolization of unpurified pancreatic tissue to the liver has been associated with a number of severe complications both experimentally and clinically (5-7) including portal hypertension. In the present study portal pressures were significantly lower following intraportal embolization of pancreatic microfragments purified by cryopreservation. Vasoactive substances and thrombogenic materials, released from exocrine cells contaminating the graft, have generally been implicated as the cause of portal hypertension under these circumstances (5,26). The significant fall in amylase concentration seen reflects the loss of exocrine tissue in the graft following cryopreservation and resulted in a reduced load of vasoactive and thrombogenic materials being presented to the portal system, explaining the significant fall in portal vein pressure following engraftment. The degree of portal hypertension seen using cryopreserved tissue was never severe enough to induce small bowel infarction as occurred in 1 dog transplanted with fresh tissue.

The occurrence of severe systemic hypotension following intraportal infusion of fresh pancreatic tissue has been described and is attributed to vasoactive kinins release from exocrine tissue in the graft (27). The greater degree of such contaminants in the fresh graft may explain the 2 sudden deaths observed following engraftment with fresh pancreatic tissue. No such deaths were observed following intraportal infusion of cryopreserved tissue.

In summary, purification of canine pancreatic microfragments by cryopreservation is demonstrated by a greater than 3-fold rise in the

insulin/amylase ratio. The function of cryopreserved autografts in the liver was similar to that of freshly prepared tissue with the advantage of significantly reduced portal hypertension during intraportal embolization. Similar results using human tissue would provide a purified islet cell preparation associated with fewer engraftment complications and should allow for safer and more successful clinical trials in the future.

TABLE II-1. Insulin amylase ratio.

	n	Spun volume (mL)	Insulin content (mU/mg)	Amylase content (IU/mg)	I:A ratio
Pancreas	6	62.3±6.3*	3.39±0.78	3.01±0.32	1.2±0.3
Processed	6	18.6±2.0	1.79±0.15	1.74±0.13**	1.0±0.1***
Post-cryo	6	13.7±1.5	2.19±0.20	0.68±0.09**	3.7±0.8***

mean±SEM
* weight (g)
** $p<0.001$
*** $p<0.01$

Table II-2. Autograft survival.

	n	Survival 1 month	Normoglycemia 1 month	Graft failure	Days Survival* (mean±SEM)
Fresh liver	6	2	2	1	3.0±1.2
Cryo liver	6	5	3	3	20.0

* of those not surviving to 1 month

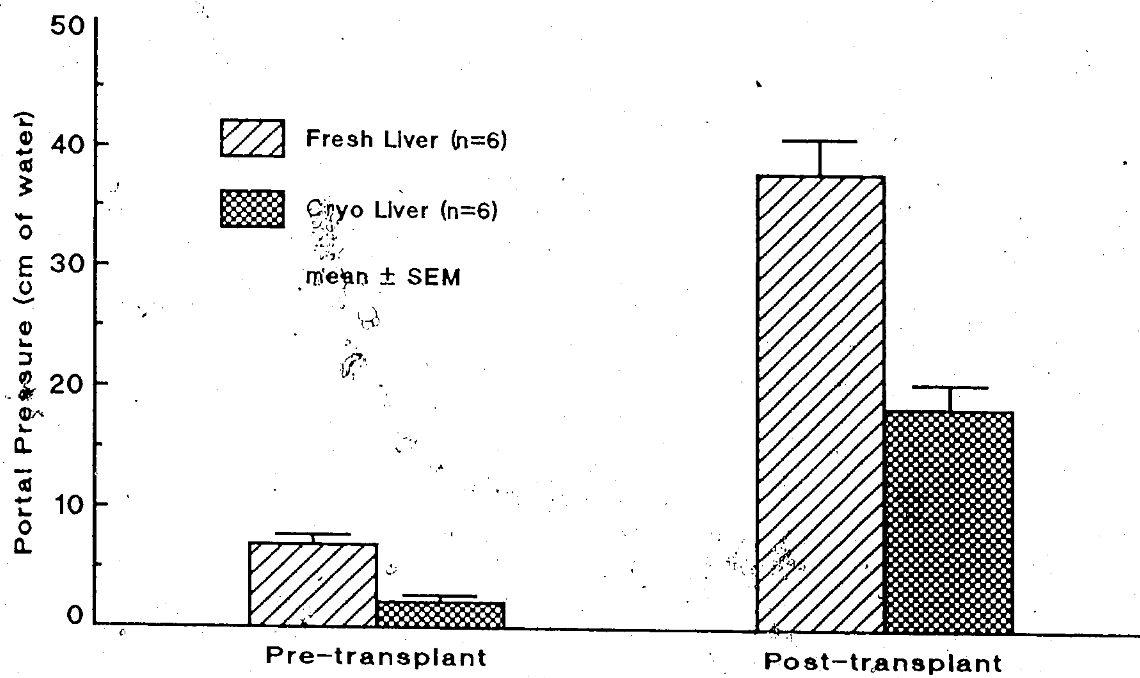


FIGURE II-1: Intraportal embolization of cryopreserved pancreatic fragments resulted in a significantly lower rise in portal venous pressure compared with freshly-prepared tissue ($p<0.01$).

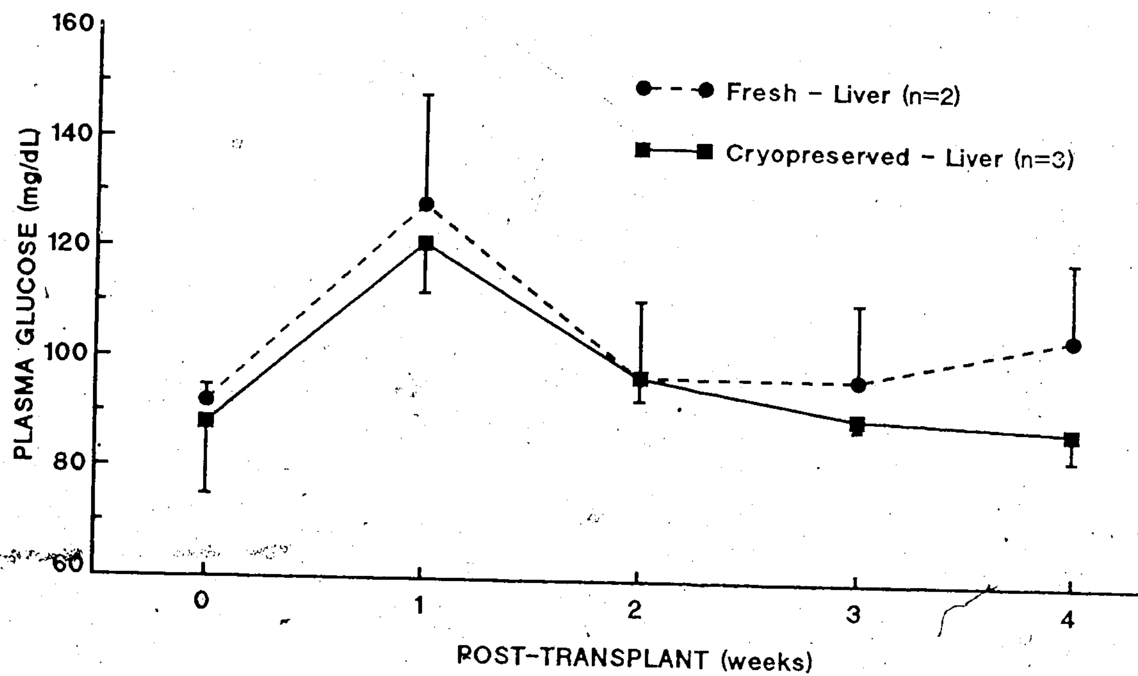
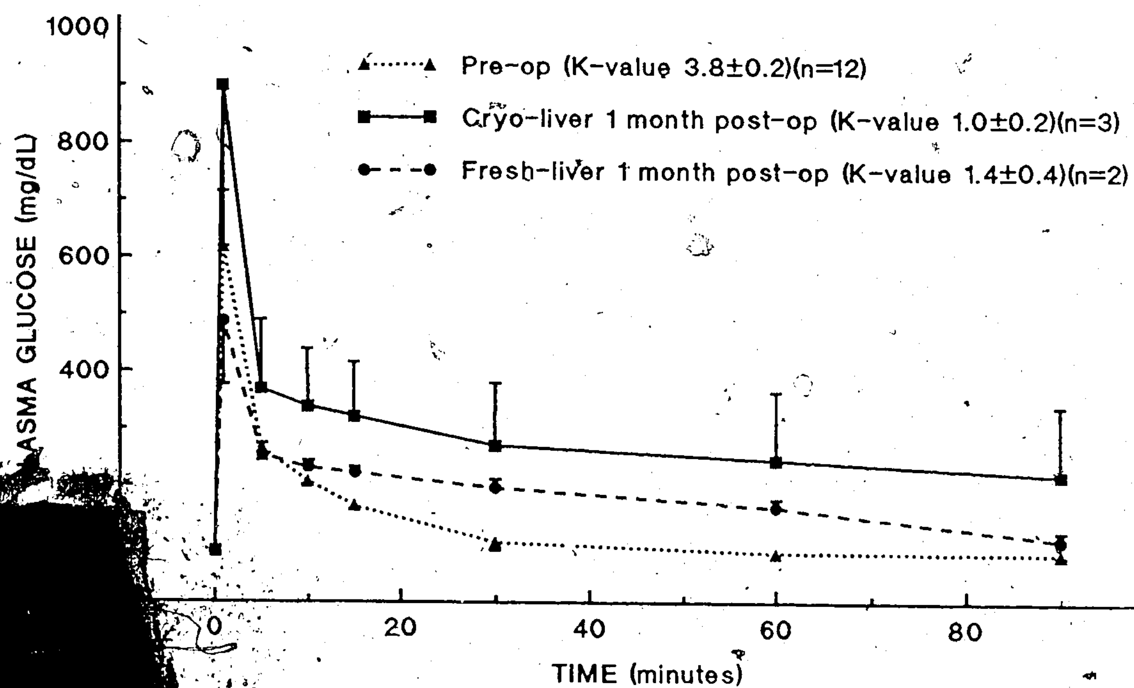


FIGURE II-2: Fasting plasma glucose following transplantation of fresh and cryopreserved pancreatic fragments in dogs normoglycemic one month after engraftment.



RE II-3: Results of intravenous glucose tolerance test performed eratively and 1 month following autotransplantation.

REFERENCES

1. Mauer SM, Sutlerland DER, Steffes MW, Leonard RJ, Najarian JS, Michael AF, Brown DM. Pancreatic islet transplantation effects on the glomerular lesions of experimental diabetes in rats. Diabetes 23(9):748, 1974.

2. Mauer SM, Steffes MW, Sutherland DER, Najarian JS, Michael AF, Brown DM. Studies of the rate of regression of the glomerular lesions in diabetic rats treated with pancreatic islet transplantation. Diabetes 24(3):280, 1975.

3. Gray BN, Watkins E. Prevention of vascular complications of diabetes by pancreatic islet transplantation. Arch Surg 111:254, 1976.

4. Mirkovitch V, Campiche M. Intrasplenic autotransplantation of canine pancreatic tissue: maintenance of normoglycemia after total pancreatectomy. Eur Surg Res 9:173, 1977.

5. Mehigan DG, Bell NR, Zuidema GD, Eggleston JC, Cameron JL. Disseminated intravascular coagulation and portal hypertension following pancreatic islet autotransplantation. Ann Surg 191(3):287, 1980.

6. Walsh TJ, Eggleston JC, Cameron JL. Portal hypertension, hepatic infarction and liver failure complicating pancreatic islet autotransplantation. Surgery 91:485, 1982.

7. Memsic L, Busuttil RW, Traverso LW. Bleeding esophageal varies and portal vein thrombosis after pancreatic mixed cell autotransplantation. Surgery 91:485, 1982.

8. Sutherland DER, Kendal D. Clinical pancreas and islet transplant registry report. Trans Proc 17(1):307, 1985.

9. Rajotte RV, Scharp DW, Downing R, Preston R, Molnar GD, Ballinger WF, Greider MH. Pancreatic islet banking: the transplantation of frozen-thawed rat islets transported between centers. Cryobiology 18:357, 1981.

10. Rajotte RV, Warnock GL, Kneteman NM. Cryopreservation of insulin producing tissue in rats and dogs. World J Surg 8:179, 1984.

11. Rajotte RV, Warnock GL, Bruch LC, Procyshyn AW. Transplantation of cryopreserved fresh rat islets and canine pancreatic fragments: comparison of cryopreservation protocols. Cryobiology 20:169, 1983.

12. Hinshaw DB, Jolley WB, Hinshaw DB, Kaiser JE, Hinshaw K. Islet autotransplantation after pancreatectomy for pancreatitis with a new method of islet preparation. Am J Surg 142:118, 1981.

13. Cobb LF, Merrel RC. Total pancreatectomy in dogs. J Surg Res 37(3):235, 1984.

14. Warnock GL, Rajotte RV, Procyshyn AW. Normoglycemia after reflux of islet containing fragments into the splenic vascular bed in dogs. Diabetes 32(5):452-458, 1983.

15. Matas AJ, Sutherland DER, Steffes MW, Najarian JS. Short-term culture of adult pancreatic fragments for purification and transplantation of islets of Langerhans. Surgery 80(2):183-191, 1976.

16. Leibo SP, Mazur P. In: Methods in Mammalian Reproduction (Daniel JC, Jr. ed.). Academic, Orlando, FL, p. 179, 1979.

17. Morgan CR, Lazarow A. Immunoassay of insulin: two-antibody system. Diabetes 12:115, 1983.

18. Sutherland DER, Matas AJ, Steffes MW, Najarian JS. Infant human pancreas: a potential source of islet tissue for transplantation. Diabetes 25:1123, 1976.

19. Moorehouse JA, Grahame CR, Rosen NJ. Relationship between intravenous glucose tolerance and the fasting blood glucose level in healthy and in diabetes subjects. J Clin Endocrin 24:145, 1964.

20. Rajotte RV, Mazur P. Survival of frozen-thawed fetal rat pancreases as functions of the permeation of dimethyl sulfoxide and glycerol, warming rate and fetal age. Cryobiology 18:17, 1981.

21. Matas AJ, Sutherland DER, Kretschmer G, Steffes MW, Najarian JS. Pancreatic tissue culture: depletion of exocrine enzymes and purification of islets for transplantation. Trans Proc 9(1):337, 1977.

22. Nason RW, Rajotte RV, Procyshyn AW, Pederson JE. Purification of canine pancreatic islet cell grafts with radiation. Trans Proc 18(1):174, 1986.

23. Sommers SC. In: Pathology of Irradiation (Berdjio CC, ed.). Williams and Wilkins, Baltimore, MD, p. 434, 1971.

24. Scharp D. Isolation of transplantation of islet tissue. World J Surg 8:143, 1984.

25. Alejandro R, Cutfield R, Shienvold FL, Latif Z, Mintz D. Successful long-term survival of [illegible]creatic islet allografts in spontaneous or pancreatectomy-in[illegible]betes in dogs. Cyclosporine-induced immune unrespons[illegible]abetes 34:825, 1985.

26. Traverso WL, [illegible] RG. Hemodynamic measurements after administration of [illegible] and/or heparin during pancreatic cell autotransplantation in dog, pig and monkey. Ann Surg 195:479, 1982.

27. Torres LE, Traverso WL, Yound ZS. Intraoperative hemodynamic changes in patients undergoing mixed-cell intraportal autotransplantation of pancreatic tissue. Anesthesiology 53:427, 1980.

III

COMPARISON OF SITES FOR TRANSPLANTATION OF CANINE PANCREATIC MICROFRAGMENTS[1]

Experimental transplantation of islets of Langerhans has been highly successful (1). This technique can not only normalize the acute manifestations of diabetes mellitus, but has been shown to prevent or reverse the chronic microvascular complications as well (2-4). Clinical trials of islet cell transplantation have not been successful (5). One of the issues that remains to be clarified is that of the optimal site for engraftment of pancreatic islet tissue. The superiority of intraportal embolization has been documented in rodent studies (6). The spleen, liver and more recently, the renal subcapsular region have been used successfully for engraftment of islet containing tissue in dogs (7-10). The degree of function and the incidence of transplant related complications varies depending on the recipient site used. In the present study we compare the function and safety of collagenase-dispersed pancreatic fragments implanted into the spleen, liver and kidney of totally pancreatectomized dogs.

MATERIALS AND METHODS

<u>Experimental design</u>

In 28 dogs, total pancreatectomy was performed 1 week after intravenous glucose tolerance test (ivGTT). Six dogs were maintained as

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[1] A version of this chapter has been accepted for publication: Evans MG, Warnock GL, Rajotte RV. Diabetes Research 1988.

apancreatic controls. Pancreatic autografts were transplanted to the spleen by venous reflux (8) in 10 dogs, to the liver by portal embolization in 6 and injected under the kidney capsule in another 6. Portal pressures were measured before and after embolization to the spleen and liver. Autograft function was assessed by fasting plasma glucose (PG) determinations daily for the first week and weekly thereafter. A second ivGTT was performed 1 month following transplantation. Liver enzymes, bilirubin and renal function were determined preoperatively and following transplantation at 1, 7 and 28 days. A coagulation profile was determined prior to, 2 h, 2 days and 7 days after transplantation to the liver and spleen. Animals were sacrificed when they lost 25% of preoperative weight in face of hyperglycemia (PG>150 mg/dL).

Animals and Care

Twenty-eight mongrel dogs of both sexes weighing 17-26.3 kg were studied. Surgical procedures were carried out under general anesthesia with sodium pentobarbital, 30 mg/kg/body weight. The dogs were weighed weekly and permitted unrestricted exercise twice daily. Full diet consisted of 360 g of meat (Dr. Ballard's, Nabisco, Toronto, ON) and 600 g of Burger Bits (Pow R Pac, Swifts, Calgary, AB) daily. On postoperative day 1 hydration was maintained with 1 L of normal saline injected subcutaneously. On postoperative day 2 water was allowed, on day 3 a meat diet was offered and on day 4 full diet resumed. Each meal was supplemented with 8 capsules of Cotazym (Organon Canada Ltd., Montreal, PQ). Prophylactic antibiotics, Derapen-C (Ayerst, Montreal, PQ) 3-4 mL, were administered at the onset of surgery, and on postoperative day 1.

Pancreatectomy

The dogs were anesthetized with sodium pentobarbital (30 mg/kg) then total pancreatectomy was performed (11). Through an upper midline incision the entire pancreas was mobilized with the 4 major vascular connections preserved. Both branches of the pancreatic duct were cannulated (PE90 polyethylene tubing) in situ. Finally, the blood vessels were clamped, ligated, and the gland removed. Immediately the pancreas was weighed and ducts distended by injection of Hank's balanced salt solution (HBSS, Gibco, Chagrin Falls, OH) of approximately 60 mL into the horizontal limb and 20 mL into the vertical limb. The gland was immersed in HBSS at 4°C and transferred to the laboratory for graft preparation. The abdomen was closed in the 6 dogs maintained as apancreatic controls. During in-vivo studies the dogs were kept under general anesthesia during graft preparation.

Graft Preparation

The graft was prepared as previously reported from this lab (8). Briefly the cannulas, were perfused with chilled Hank's balanced salt solution (HBSS) for 10 min at a constant pressure of 300 mm Hg. The perfusate was then changed to a solution of 0.4% collagenase (Type V, 490 U/mg, Sigma Chemicals, St. Louis, MO) in HBSS at 37°C. The gland was suspended on a 60-μm mesh above the reservoir of collagenase from which the enzyme was reperfused. When digestion was judged complete the gland was bisected and transferred to 2 jars containing 50 mL of enzyme-stop solution consisting of 4°C HBSS supplemented with 2% trasylol (Miles Pharmaceuticals, Elkart, Indiana), penicillin 100 U/mL and streptomycin 100 ug/mL. The tissue was minced for exactly 90 seconds in a mechanical mincer (12). The supernatant fluid was

discarded and the preparation resuspended in fresh enzyme-stop solution and transferred to an Ehrlenmeyer flask. Dissociation was accomplished by shaking the flask vigorously in an Evapomix for 10 min at 4°C, followed by filtration through a 400-μm screen. Residual unfiltered tissue was returned to the Ehrlenmeyer flask and passed through the dissociation process again. Finally, the preparation was washed 3 times in enzyme-stop solution and the filtrate was centrifuged at 2000 rpm for 20 seconds and supernatant fluid removed. The pellet of islet tissue was resuspended in 30-40 cc of enzyme-stop solution prior to immediate autotransplantation. These procedures were performed in a laminar flow hood with the time for graft preparation being approximately 2 h.

Transplantation Procedure

The graft, resuspended in 30 mL of chilled Hank's balanced salt solution (HBSS) with 2% Trasylol, penicillin and streptomycin, was isografted immediately following graft preparation. Intrasplenic injection was by venous reflux (8). Briefly, the spleen was delivered into the operative field and PE90 polyethylene tubing inserted by venotomy into superior and inferior terminal splenic veins advancing the tubing to the hilus and not into the spleen. Vascular clamps were applied to the splenic pedicle. The graft suspension was injected into the cannulae over a period of 5 min to allow tissue to reflux into venous sinusoids. The cannulas were then withdrawn and veins ligated. The vascular clamps were released 5 min after injection of the graft was completed.

Liver engraftment was performed by intraportal embolization. Silastic tubing (0.040 inches ID, Dow Corning, Midland MI) was introduced via the gastroduodenal vein remnant and advanced into the

portal vein, with a 3-way stopcock attached to a manometer to allow monitoring of portal venous pressure. Sodium heparin (Allen and Hansburgs, Glaxo Canada Ltd, Toronto, ON), 150 United States Pharmacopeia U/kg was given intravenously. Pancreatic tissue was injected in 6 equal aliquots, each infused over 5 min at intervals of 5 min with a total infusion time of 1 h. The silastic catheter was flushed with 5 mL of saline containing 1000 USP units of sodium heparin per liter after each aliquot.

Prior to engraftment under the renal capsule the left kidney was first mobilized by dividing the peri renal fascia. The capsule at the lower pole was then punctured and cannulated (PE90 polyethylene tubing). The graft was resuspended in 15 mL of HBSS with 2% Trasylol, penicillin and streptomycin and was infused slowly while the catheter was manipulated in multiple directions to distribute the graft under approximately 75% of the subcapsular space. Manual pressure was applied to the puncture site for 5 min following injection.

Blood Indices

Fasting plasma glucose (PG mg/dL) was measured by the glucose oxidase method using the Beckman Glucose Analyzer II (Beckman Instruments, Brea, CA).

For intravenous glucose tolerance tests (ivGTT), saphenous veins were cannulated, glucose (0.5 g/kg body weight) was injected and blood collected at 0, 1, 5, 10, 15, 30, 60 and 90 min for assays of glucose . The K value (13), a measure of the decline in glucose level (%/min), was determined from glucose levels at 5, 10, 15 and 30 min. Alkaline phosphatase (ALk Phos) serum glutamate oxalacetic transaminase (SGOT), lactate dehydrogenase (LDH), bilirubin, blood urea nitrogen (BUN), and

creatine were measured using the Multistat III analyzer (Instrumentation Laboratory, Lexington, MA). Prothrombin times (PT) and partial thromboplastin times (PTT) were assayed using the BBL-Fibrometer (Becton-Dickinson, Missisauga, ON) Platelet counts were performed on a TOA Automatic Platelet Counter, Model PL-100 (TOA Medical Electronics Co., Kobe, Japan) with adjustment for the size of dog platelets. Fibrin degradation products (FDP) were measured using the Thrombo-Wellco test (Wellcome Diagnostics, Dartford, England).

Analysis of Data

All results are expressed as a mean±SEM. The data were analyzed using the Student's t-test for paired or unpaired data as applicable. Differences are significant when $P<0.05$ unless stated otherwise.

RESULTS

Portal Pressure (Figure III-1)

Portal pressures (cm H_2O) after the infusion of pancreatic fragments to the spleen and liver are shown. Following intraportal embolization the portal pressure rose from 6.9±0.87 to 38±3.02 ($P<0.001$). Intrasplenic engraftment was not associated with a significant change in portal pressure.

Survival of Dogs (Figure III-2)

All 6 apancreatic dogs became severely diabetic surviving 6.5±0.75 days. Of the 10 dogs receiving intrasplenic autografts 9 were normoglycemic at 1 month. One dog became hyperglycemic (plasma glucose [PG] 350 mg/dL) on day 4 following engraftment and remained so for the remainder of follow-up. Following intraportal embolization, 2 of 6 dogs remained normoglycemic at 1 month. One died due to small bowel

infarction; 2 died suddenly within 24 h of engraftment without obvious cause, and a fourth died due to persistent diabetes 7 days following the transplant with a PG of 678 mg/dL. None of the dogs engrafted under the kidney capsule became normoglycemic. One dog died within 24 h of engraftment before PG was measured, while another died on the eighth post-transplant day (PG 436 mg/dL), the remaining 4 dogs became progressively hyperglycemic.

Autograft Function

Fasting PG in dogs maintained for follow-up is shown in figure III-3. Following pancreatectomy control dogs became rapidly hyperglycemic with a PG of 428±45 at death. Function is similar in the recipients of intrasplenic and intraportal grafts, while recipients of renal capsule grafts demonstrate progressive hyperglycemia. The pretransplant K value of all dogs was 3.4±0.2 which fell significantly at 1 month to 1.3±0.2 for recipients of intrasplenic grafts ($p<0.01$) and 1.4±0.4 for successful intraportal grafts ($p<0.01$).

Liver Function (Table III-1)

Alkaline phosphatase rose significantly in recipients of intrasplenic ($p<0.001$) intraportal ($p<0.01$) and renal capsule ($p<0.05$) grafts within 24 h. The differences between groups at 24 h were not significant. Values returned to preoperative levels in all groups by 1 month. Serum GOT values were significantly elevated following intrasplenic ($p<0.001$), intraportal ($p<0.01$) and renal capsule grafts ($p<0.001$) 24 h following transplantation. The differences between groups at 1 day were not significant and values returned to preoperative levels in all groups by 1 month. Lactate dehydrogenase rose significantly following engraftment in the spleen ($p<0.05$) and liver

($p<0.01$) one day following transplant, but was not affected by renal capsule engraftment. The differences between groups observed at 24 h were not significant. At 1 month values have returned to preoperative levels in both groups. Despite these transient elevations in liver enzymes following transplantation, plasma bilirubin was not affected.

Renal Function

Blood urea nitrogen and serum creatinine measured preoperatively in recipient dogs were 13.6±1.4 and 1.0±0.07 respectively. No significant change in renal function was noted following engraftment regardless of the site used.

Coagulation Profile (Table III-2)

Coagulation parameters were measured in dogs receiving intrasplenic and intraportal grafts. The number of dogs (n) is the same for other parameters as shown for PT. Prothrombin time was not significantly affected by transplantation to either organ. Significant prolongations in PTT occurred 2 h following intraportal embolization of graft. Platelet counts were significantly depressed 2 days following intrasplenic engraftment. Both abnormalities were transient, having returned to normal by 1 week. Isolated elevation of FDP above 40 mg/L was noted in 1 dog 2 days after receiving an intrasplenic graft and in one other 1 week after intraportal embolization.

DISCUSSION

The spleen (7,8), liver (9) and recently the kidney (10) have been used successfully as recipient sites for canine pancreatic microfragments. The findings of this study are consistent with that reported by other authors, supporting the reliability of the spleen as a

recipient site for pancreatic microfragments. Kretschmer et al reversed hyperglycemia for up to 10 weeks in 20 of 21 dogs receiving an intrasplenic graft of collagenase dispersed pancreatic tissue (14). Warnock demonstrated similar findings normalizing plasma glucose (PG) in 9 of 13 dogs, autografted to the spleen by splenic vein reflux (8). More recently Hesse and Sutherland autografted pancreatic microfragments to the spleen, by venous reflux as well, and reversed diabetes in 10 of 14 dogs rendered diabetic by total pancreatectomy and followed for 2 to 6 weeks (15). In the present study, as in those previously mentioned, glucose tolerance is consistently impaired with K values at 1 month significantly lower than preoperative values. Whether the reduced function of intrasplenic grafts is a quantitative or qualitative defect is not known; however, it has been shown that over time the function of intrasplenic autografts, as measured by K values, improves and stabilizes without evidence of fatigue for up to 6 months (16).

In contrast to the success of intrasplenic transplants, only 2 of 6 dogs receiving intraportal pancreatic microfragments became normoglycemic, both surviving to 1 month. One dog died due to graft failure and complications were a significant problem with other dogs. One dog died 4 days post-transplant due to complete small bowel infarction secondary to severe and persistent portal venous hypertension following graft infusion. Two dogs died suddenly within 24 h following engraftment. Documentation of graft function was not achieved before death and autopsy did not reveal the cause of death in either of these dogs. Post-transplant fasting PG levels were similar in the remaining two dogs to those of dogs engrafted to the spleen, as were K values determined by ivGTT at 1 month. This suggests that the function of

canine pancreatic fragments engrafted to either the spleen or liver is comparable.

Intraportal infusion of canine pancreatic microfragments has previously been reported. Kretchmer and associates produced normoglycemia in 3 of 10 dogs transplanted with collagenase digested pancreatic tissue (9). In contrast to the present study, graft failure was responsible for the death of all 7 unsuccessful recipients.

Kolb et al (17) successfully reversed diabetes in 5 of 5 dogs by intraportal embolization of pancreatic fragments, and Horaguchi and Merrell (18) had similar results in 3 of 5 dogs.

The subcapsular region of the canine kidney was demonstrated to be a potential recipient site by Toledo-Pereyra and associates when they reversed diabetes in pancreatectomized dogs with allografts prepared without collagenase digestion and mechanically dispersed (10). Normoglycemia was maintained without immunosuppression or with azathioprine (2.5-5 mg/kg/day) for greater than 6 months suggesting that the renal subcapsular region may also be an immunoprivileged site. In contrast, Hesse and Sutherland were unable to produce normoglycemia in 11 dogs autografted with collagenase digested pancreatic microfragments (15). The findings of the present study are in keeping with those of Hesse and Sutherland. None of the 6 dogs engrafted under the renal capsule maintained normoglycemia. It routinely took 2 or 3 days for fasting sugars to reach diabetic levels in contrast to apancreatic controls suggesting some minimal graft function initially. All dogs were hyperglycemic by the end of 1 week with fasting glucose values in the range seen with apancreatic controls. As in the study of Hesse and Sutherland, the survival of the dogs was prolonged compared with

apancreatic animals (with 4 of 6 dogs surviving 30 days) suggesting residual β cell function. In the present study islets were not observed in the tissue under the renal capsule. Histologically the specimens consisted only of necrotic debris and some fibrous tissue.

These results contrast dramatically with those of Toledo-Pereyra et al, who used a graft prepared solely by mechanical means without the use of collagenase digestion and performed allografts rather than transplanting autologous tissue, as in the present study and that of Hesse and Sutherland. Although differences in experimental protocol may exclude direct comparison with that of Toledo-Peryra, the results of the present study would support the conclusion that the renal subcapsular region is not an appropriate site for the engraftment of pancreatic microfragments.

The safety of engraftment of islet containing tissue was also examined in the current study. Portal hypertension in varying degrees has been described both in dogs and in clinical trials following the embolization of pancreatic tissue into the portal vein (9,17,19-21,28) and DuToit documented portal hypertension following inadvertent embolization to the liver of intrasplenic pancreatic tissue (22). In this study, during the infusion of pancreatic microfragments into the splenic veins, vascular clamps were placed occluding the hilum of the spleen for 5 min following engraftment, then removed. There was a slight but insignificant rise in portal pressures following engraftment. Although measurements of this kind following intrasplenic transplantation, have not previously been reported in the literature, these results are in clear contrast to the degree of portal hypertension well documented to occur during intraportal embolization (9,17,19-21).

Following intraportal infusion, portal hypertension was a consistent finding in all dogs in this study and was directly responsible for the death of 1. The volume infused was the same as that grafted into the spleen suggesting that the elevation in portal pressure is specific for the intraportal route of engraftment. Kretschmer et al (9) documented transient elevation in portal pressures in dogs, but greater elevations have also been described leading to severe complications such as portal vein thrombosis, bleeding esophageal varices, (23) hepatic infarction and death (20).

Despite the clear association of portal hypertension with intraportal embolization, liver function abnormalities occurred in all groups receiving pancreatic fragments. This was characterized by significant elevation of Alk. Phos. and SGOT within 24 h of engraftment in dogs of all groups, while LDH elevation occurred in dogs engrafted to the liver and spleen. These findings were transient having returned to preoperative levels by the end of 1 week in most cases and by 1 month in all dogs and were not associated with changes in serum bilirubin. Transient alterations in liver enzymes following intrasplenic and intraportal infusion of islet containing fragments has been previously described (9,17) and it has been suggested that such changes reflect hepatocellular damage following intraportal infusion either directly or subsequent to intrasplenic engraftment. However, the finding of similar transient changes in enzymes following injection under the renal capsule demonstrates that this abnormality is not specific for sites draining into the portal circulation.

Disseminated intravascular coagulation has been documented secondary to intraportal (2) and intrasplenic (24) engraftment of

pancreatic microfragments. Isolated transient abnormalities in the platelet count and FDP 2 days following intrasplenic engraftment and in the PTT and FDP 2 h and 1 week after intraportal embolization were seen. A consistent pattern of clotting abnormalities suggestive of a consumptive coagulopathy was not observed following engraftment to either the liver or spleen. The use of aprotinin in the graft preparation and heparin administered intravenously may have contributed to the lack of coagulation problems as has been suggested by others (19,25).

The occurrence of severe systemic hypotension following intraportal infusion of pancreatic tissue has been described and attributed to vasoactive substances released from contaminating exocrine tissue in the graft (26). This phenomenon may explain the 2 sudden deaths observed in dogs engrafted to the liver. No such deaths were observed in dogs following intrasplenic transplantation.

Renal function was also monitored. Blood urea nitrogen and serum creatinine were not affected following engraftment regardless of the site used.

In summary, the spleen, liver and kidney were compared as potential recipient sites for canine pancreatic fragments. In the current study 9 of 10 dogs transplanted to the spleen by venous reflux became normoglycemic without mortality or complications, suggesting that the splenic vascular bed is a reliable and safe recipient site. In contrast Intraportal infusion was successful in only 2 of 6 animals and acute complications accompanied its use. Implantation into the renal subcapsular space was associated with early graft failure in 100% of

animals suggesting that it is not an appropriate site for collagenase dispersed pancreatic fragments.

TABLE III-1. Liver function tests following transplantation*.

	Pre-op	1 day	7 day	1 month
ALK PHOS (Iu/L)				
Spleen	39±8.2 (10)	224±28 (10)	157±31 (9)	65±11 (9)
Liver	26±9.2 (6)	145±32 (4)	156±68 (3)	38±2.4 (2)
Kidney	53±9.2 (6)	234±65 (5)	275±129 (5)	242±150 (2)
SGOT (Iu/L)				
Spleen	28±1.2	261±39	33±2.5	34±2.5
Liver	26±2.9	268±57	48±7.2	38±9.5
Kidney	35±4.3	271±34	83±30	46±12
LDH (Iu/L)				
Spleen	159±20	278±42	141±37	130±41
Liver	142±22	429±77	287±55	61±16
Kidney	172±12	305±131	160±36	190±55
BILIRUBIN (mg/dL)				
Spleen	0.44±0.06	0.55±0.09	0.33±0.06	0.5±0.07
Liver	0.37±0.03	1.0±0.34	0.81±0.14	0.38±0.19
Kidney	0.82±0.14	0.82±0.42	0.6±0.12	0.45±.13

* mean±SEM

( ) number of dogs

TABLE III-2. Coagulation profile of dogs receiving intrasplenic and intraportal pancreatic fragments.

	Pre-op	2 hours	2 days	1 week
PT (sec)				
Spleen	14.5±0.02 (10)	9.8±0.34 (10)	9.6±0.13 (10)	9.8±0.24 (10)
Liver	9.5±0.02 (6)	9.7±0.08 (6)	9.5±0.01 (3)	9.5±0.03 (3)
PTT (sec)				
Spleen	14.8±0.43	15.7±0.82	16.5±0.73	14.3±0.50
Liver	15.2±1.42	24.5±0.50	19.3±1.45	16±3.0
PLATELETS ($x10^3$)				
Spleen	250±20.2	193±20.4	185±19.8	224±22.8
Liver	298±14.7	302±53.4	255±23.4	319±27.4
FDP (>40 mg/L)*				
Spleen	0	0	1	0
Liver	0	0	0	1

( ) number of dogs

* significant fibrolysis

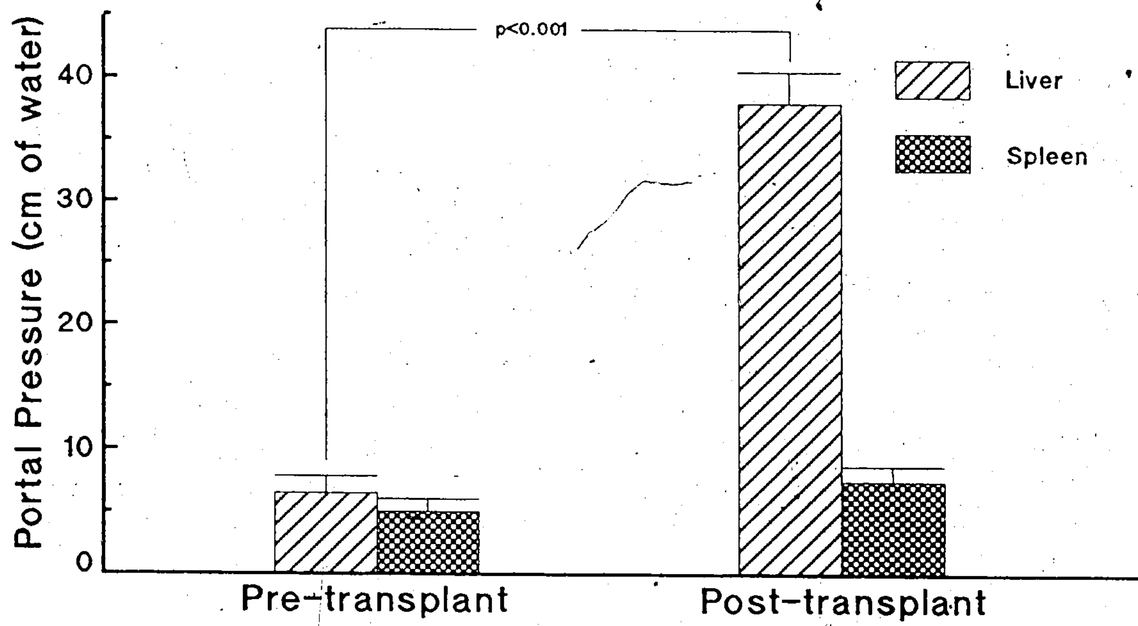

FIGURE III-1: The effect of intrasplenic and intraportal engraftment of pancreatic fragments on portal pressure.

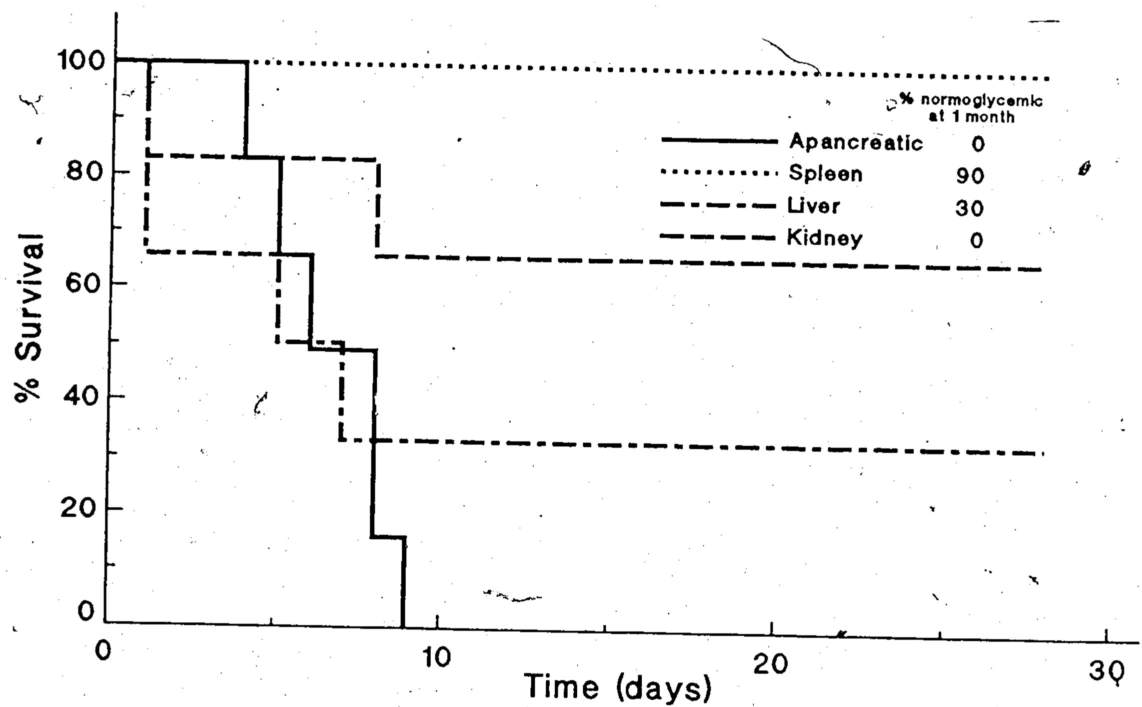

FIGURE III-2: Survival of dogs following transplantation.

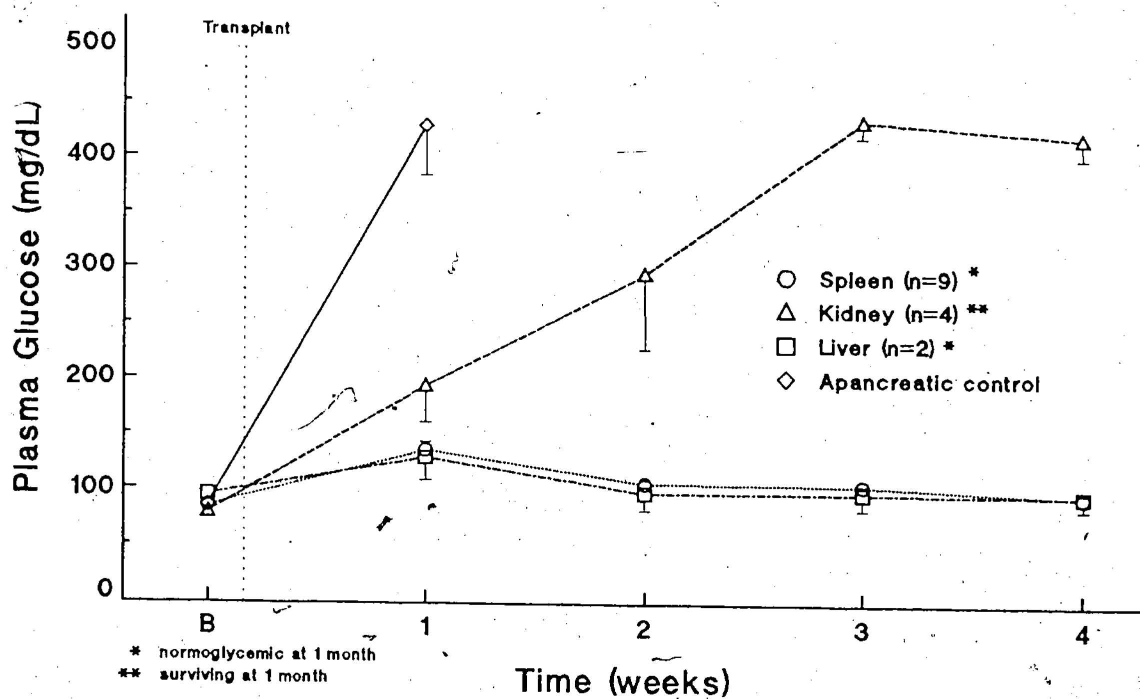

FIGURE III-3: Fasting plasma glucose of dogs following pancreatic fragment transplantation.

REFERENCES

1. Sutherland DER. Pancreas and pancreatic islet cell transplantation. I. Experimental Studies. Diabetologia, 1981; 20: 161.

2. Mauer MS, Steffes MW, Sutherland DER, Najarian JS, Michael AF, Brown DM. Studies of the rate of regression of the glomerular lesions in diabetic rats treated with pancreatic islet transplantation. Diabetes, 1975; 24 (3): 280.

3. Krupin T, et al. Ocular flourophotometry in experimental diabetes mellitus: The effect of pancreatic islet isografts. Invest Opthalmol and Visual Sci, 1979; 18: 1185.

4. Nelson J, Lacy P, Hirschberg G. Megacolon and autonomic neuropathy in diabetic rats. J Neuropath Exp Neurol, 1976; 35: 335.

5. Sutherland DER, Kendal D. Clinical pancreas and islet transplant. Registry Report. Trans Proc 1985; 17 (1): 307.

6. Kemp CB, Knight MJ, Scharp DW, Ballinger WF, Lacy PE. Effect of transplantation site on the results of pancreatic islet isografts in diabetic rats. Diabetologia, 1973; 9: 486.

7. Mirkovitch V, Campiche M. Intrasplenic autotransplantation of canine pancreatic tissue: maintenance of normoglycemia after total pancreatectomy. Eur Surg Res, 1977; 9: 173.

8. Warnock GL, Rajotte RV, Procyshyn AW. Normoglycemia after reflux of islet containing fragments into the splenic vascular bed in dogs. Diabetes, 1983; 32 (5): 452.

9. Kretschmer GJ, Sutherland DER, Matas AJ, Payne WD, Najarian JS. Autotransplantation of pancreatic fragments to the portal vein and spleen of totally pancreatecomized dogs. Ann Surg, 1978; 187 (1): 79.

10. Toledo-Peyera LH, Bandlein KO, Gordon DA, MacKenize GH, Reyman TA. Renal subcapsular islet cell transplantation. Diabetes, 1984; 33: 910.

11. Cobb LF, Merrel RC. Total pancreatectomy in dogs. J Surg Res, 1984; 37 (3): 235.

12. Matas AJ, Sutherland DER, Steffes MW, Najarian JS. Short-term culture of adult pancreatic fragments for purification and transplantation of islets of Langerhans. Surgery 80(2), 1976; 183.

13. Moorehouse JA, Grahame GR, Rosen NJ. Relationship between intravenous glucose tolerance and the fasting blood glucose level in healthy and in diabetic subjects. J Clin Endocrinol, 1964; 24: 145.

14. Kretschmer GJ, Sutherland DER, Matas AF, Steffes MW, Najarian JS. The dispersed pancreas: transplantation without islet purification in totally pancreatectomized dogs. Diabetologia, 1977; (13): 495.

15. Hesse UJ, Sutherland DER. Comparison of splenic and renal subcapsular islet autografting in dogs. Transplantation, 1986; 41 (2): 271.

16. Rajotte RV, Warnock GL, Kneteman NM. Autotransplantation of fresh and cryopreserved canine islets. Diabetes Research and Clinical Practice. XII Congress of the International Diabetes Federation, 1985, Suppl1, No. 1197, S460.

17. Kolb E, Ruckert R, Largardier F. Intraportal and intrasplenic autotransplantation of pancreatic islets in dogs. Eur Surg Res, 1977; 9: 419.

18. Horaguchi A, Merrel RC. Preparation of viable islet cells from dogs by a new method. Diabetes, 1981; 30: 455.

19. Mehigan DG, Bell NR, Zuidema GD, Eggleston JC, Cameron JL. Disseminated intravascular coagulation and portal hypertension following pancreatic islet autotransplantation. Ann Surg, 1980; 191 (3): 287.

20. Walsh TJ, Eggleston JC, Cameron JL. Portal hypertension, hepatic infarction, and liver failure complicating pancreatic islet autotransplantation. Surgery, 1982; 92: 485.

21. Traverso WL, Abou-Zamzam AM, Longmire WP. Human pancreatic cell autotransplantation following total pancreatectomy. Annals of Surgery, 1980; 193 (2), 191.

22. DuToit DF, Reece-Smith H, McShane P, Denton T, Morris PJ. Intraportal embolization of fragments during intrasplenic pancreatic autotransplantation in dogs. Transplantation, 1980; 30: 389.

23. Memsic L, Busuttil RW, Traverso LW. Bleeding esophageal varies and portal vein thrombosis after pancreatic mixed cell autotransplantation. Surgery, 1984; 95 (2), 238.

24. Miller BHR, Bewick M, Compton FJ, Needham JM, Godwin KL, Winter M. Disseminated intravascular coagulation after dispersed pancreas transplantation in dogs: causitive agents. Transplantation, 1983; 36 (3): 348.

25. Traverso WL, Gomez RG. Hemodynamic measurements after administration of aprotinin and/or heparin during pancreatic cell autotransplantation in dog, pig and monkey. Ann Surg, 1982; 195: 479.

26. Torres LE, Traverso WL, Yound ZS. Intraoperative hemodynamic changes in patients undergoing mixed cell intraportal autotransplantation of pancreatic tissue. Anaesthesiology, 1980; 53: 427.

IV

TRANSPLANTATION OF CRYOPRESERVED CANINE PANCREATIC MICROFRAGMENTS TO THE LIVER AND KIDNEY

Experimental islet cell transplantation is very successful in syngeneic strains of rodents where purified islets from multiple donors can normalize serum glucose and reverse microvascular complications in diabetic recipients (1-3). The techniques developed in the rodent model have proved ineffective for isolating sufficient islets from the compact pancreas of large mammals and humans. A partial solution to this problem, validated in the canine model, has been to eliminate the steps of purification, which reduces islet yields and collect islet-containing pancreatic fragments (4). However, the presence of contaminating exocrine and immunogenic cells in these grafts has been responsible for a number of unacceptable complications (5-7), thus precluding effective clinical trials (8).

Cryopreservation is an effective method for storage of purified islets (9) and dispersed pancreatic tissue (10,11). Furthermore, cryopreservation may destroy contaminating exocrine cells, an observation which concurs with the reputed sensitivity of exocrine tissue to cold (11,12). The ability to remove harmful exocrine tissue from dispersed pancreatic tissue would reduce complications associated with its use. The present study examines the effect of cryopreservation on pancreatic microfragment purity. Function of the cryopreserved autograft was compared with that of fresh tissue following intraportal embolization and injection under the kidney capsule in the canine model.

MATERIALS AND METHODS

Experimental Design

In 30 dogs, total pancreatectomy was performed 1 week after intravenous glucose tolerance testing (ivGTT). Six dogs were maintained as apancreatic controls. Tissue was removed from freshly prepared and cry reserved grafts for determination of insulin and amylase content. Six dogs each were engrafted by portal vein embolization and by injection under the kidney capsule. An equal number of control animals received fresh tissue by the same methods. Portal pressure was measured before and after embolization to the liver. Autograft function was assessed by fasting plasma glucose (PG) determinations daily for the first week and weekly thereafter. A second GTT was performed 1 month following transplantation. Liver enzymes, bilirubin and renal function were assessed preoperatively and following transplantation at 1, 7 and 28 days. A coagulation profile was measured prior to and 2 h, 2 days and 7 days after intraportal embolization. Animals were sacrificed when they lost 25% of preoperative weight in the face of hyperglycemia (PG>150 mg/dL).

Animals and Care

Thirty mongrel dogs of both sexes weighing 17-26.3 kg were studied. Surgical procedures were carried out under general anesthesia with sodium pentobarbital, 30 mg/kg/body weight. The dogs were weighed weekly and permitted unrestricted exercise twice daily. Full diet consisted of 360 g of meat (Dr. Ballard's, Nabisco, Toronto, ON) and 600 g of Burger Bits (Pow R Pac, Swifts, Calgary AB) daily. On postoperative day 1 hydration was maintained with 1 L of normal saline injected subcutaneously. On postoperative day 2 water was allowed, on

day 3 a meat diet was offered and on day 4 full diet resumed. Each meal was supplemented with 8 capsules of Cotazym (Organon Canada Ltd., West Hill, ON). Prophylactic antibiotics, Derapen-C (Ayerst, Montreal, PQ) 3-4 mL, were administered at the onset of surgery and on postoperative day 1. Until cryopreserved grafts were thawed and reimplanted, daily subcutaneous injections of 6-8 units of NPH insulin (Connaught Laboratories, Willowdale, ON) were administered.

Pancreatectomy

Through an upper midline incision total pancreatectomy was performed (13). Both branches of the pancreatic duct were cannulated (PE90 polyethylene tubing) in situ, then the blood vessels were clamped, ligated and the gland removed. Immediately the pancreas was weighed and ducts distended by injection of Hank's balanced salt solution (HBSS, Gibco, Chagrin Falls, OH) approximately 60 mL into the horizontal limb (body and tail) and 20 mL into the vertical limb (uncinate process). The gland was immersed in HBSS at 4°C and transferred to the laboratory for graft preparation. When transplanting fresh tissue an assistant maintained the dogs under general anesthesia during graft preparation. Cryopreserved grafts were transplanted 24-48 h later at a second laparotomy.

Graft Preparation

The graft was prepared by a method previously reported from this lab (14). Briefly, the cannulas were connected to a perfusion apparatus and flushed with chilled (4°C) HBSS at a pressure of 300 mmHg. The perfusate was then changed to a solution of 0.4% collagenase (Type V, 490 U/mg, Sigma Chemicals, St. Louis, MO) in HBSS at 37°C. The gland was suspended on a 60-μm mesh above the reservoir of collagenase from

which the enzyme was reperfused. When digestion was judged complete the gland was bisected and transferred to 2 jars containing 50 mL of enzyme-stop solution consisting of 4°C HBSS supplemented with 2% trasylol (Miles Pharmaceuticals, Elkart, IN), penicillin 100 U/mL and streptomycin 100 μg/mL. The tissue was minced for exactly 90 seconds in a mechanical mincer (15). The supernatant fluid was discarded and the preparation resuspended in fresh enzyme-stop solution and transferred to an Ehrlenmeyer flask. Dissociation was accomplished by shaking the flask vigorously in an Evapomix for 10 min at 4°C, followed by filtration through a 400-μm screen. Residual unfiltered tissue was returned to the Ehrlenmeyer flask and passed through the dissociation process again. Finally, the preparation was washed x3 in enzyme-stop solution. The filtrate was centrifuged at 2000 rpm for 20 seconds and supernatant fluid removed. The pellet of islet tissue was resuspended in 30-40 cc of enzyme-stop solution prior to immediate autotransplantation (12 dogs). Prior to cryopreservation (12 dogs) the pellet was resuspended by doubling its volume in tissue culture media Medium 199 (Gibco, Chagrin Falls, OH) supplemented with 2% trasylol, 10% fetal calf serum (FCS), penicillin 200 U/mL and streptomycin 200 μg/mL. These procedures were performed under a laminar flow hood with the time for graft preparation being approximately 2 h.

Cryopreservation

Freezing and thawing were by methods previously reported (11). Briefly the tissue pellet was resuspended at 22°C in Medium 199 with 10% FCS, 2% aprotinin, penicillin 200 U/mL and streptomycin 200 μg/mL (V/V). A volume equal to that of the original tissue pellet of 2M dimethyl sulfoxide (Me_2SO) in the same Medium 199 mixture was then added at 25°C.

After 5 min another volume of 2M (Me_2SO) solution was then added and shaking continued for 25 min. Four volumes of 3M (Me_2SO) solution were added (final Me_2SO concentrate in islet bearing solutions was 2M) followed by shaking at 25°C for 15 min.

The tissue suspension (0.5 mL tissue in 4 mL suspension) was then aliquoted to 16x125 mm glass KIMAX (Owens, Illinois) test tubes at 4°C and after 10 min the tubes were transferred to an ethanol bath at -7.2°C to supercool for 5 min. The suspension was then nucleated by contacting the tube with stainless steel tubing through which liquid (N_2) was pumped. Fifteen min were allowed for release of the latent heat of fusion, then the tubes were transferred to evacuated dewars filled with ethanol and surrounded by liquid N_2 for cooling at 0.25°C/min (16). When the tissue reach -40°C it was plunged into liquid N_2 (-196°C) for storage.

Thawing Procedure

After 24-72 h the tissue was thawed by agitation in a water bath at 37°C (150°C/min). Supernatant was aspirated after centrifugation (1500 rpm) and 2.5 mL of 0.75 mol/L sucrose in Medium 199 solution was added to each aliquot. After equilibration of the preparation 30 min the sucrose was di[illegible]ted in 4 concurrent steps of 5 min each by the addition of Medium 199 with 10% FCS, 2% trasylol, penicillin and streptomycin while shaking at 22°C. The mixture was centrifuged, the pellet washed, spun and resuspended to 30 mL with Medium 199 solution for transplanting.

Transplantation Procedure

Liver engraftment was performed by intraportal embolization. Silastic tubing (0.040 inches ID, Dow Corning, Midland, MI) was

introduced via the gastroduodenal vein and advanced into the portal vein with a 3-way stopcock attached to a monometer to allow monitoring of portal enous pressure. Sodium Heparin (Allen and Hansburgs, Glaxo Canada Ltd., Toronto, ON), 150 United States Pharmacopeia (USP) U/kg was given intravenously. Pancreatic tissue was given in 6 equal aliquots each infused over 5 min, at intervals of 5 min with a total infusion time of 1 h. The Silastic catheter was flushed with 5 cc of saline containing 1000 USP units of sodium heparin per liter after each aliquot. Prior to engraftment under the renal capsule the left kidney was mobilized by dividing the perirenal fascia. The capsule at the lower pole was then punctured and cannulated (PE90 polyethylene tubing). The graft, resuspended in 15 mL of HBSS with 2% trasylol, penicillin and streptomycin was infused slowly while the catheter was manipulated in multiple directions to distribute the graft under approximately 75% of the subcapsular space. Manual pressure was applied to the puncture site for 5 min following injection.

Tissue Assays

Samples of freshly prepared and cryopreserved grafts were weighed homogenized and sonicated in 5 mL of chilled 2% acid (H_2SO_4): 76% ETOH. Insulin was extracted over 24 h at 4°C. Samples were neutralized and diluted in saturated sodium bicarbonate (Gibco, Grand Island, NY). Insulin was measured by double-antibody radioimmunoassay (17) using Insulin RIA kits (Pharmacia, Uppsala, Sweden) and human insulin standards (WHO International Lab for Biol. Standards) and amylase with Dri-STAT Amylase-DS reagent (Beckman Instruments, Carlsbad, CA). The ratio of insulin to amylase content was determined as a measurement of purity. The comparison was made under the assumption that tissue

insulin and amylase content are proportional to pancreatic B-cell and exocrine tissue mass respectively (18).

Blood Indices

Fasting PG (PG mg/dL) was measured by the glucose oxidase method using the Beckman Glucose Analyzer 2 (Beckman Instruments, Brea, CA). For ivGTT saphenous veins were cannulated, glucose (0.5 g/kg body wt) was injected and blood collected at 0, 1, 5, 10, 15, 30, 60, 90 min for assays of glucose. The K value (19), a measure of the decline in glucose level (percent/min) was determined from glucose levels at 5, 10, 15, and 30 min. Alkaline phosphate (Alk. Phos.) serum glutamate oxalacetic transaminase (SGOT), lactate dehydrogenase (LDH, bilirubin, blood urea nitrogen (BUN) and serum creatine were measured using the Multistat III analyzer (Instrumentation Laboratory, Lexington, MA). Prothrombin times (PT) and partial thromboplastin times (PTT) were assayed using the BBL-fibrometer (Becton-Dickinson, Mississauga, ON). Platelet counts were performed on a TOA Automatic Platelet Counter, Model PL-100 (TOA Medical Electronics Co. Kobe, Japan) with adjustment for the size of dog platelets. Fibrin degradation products (FDP) were measured using the Thrombo-Wellco test (Wellcome Diagnostics, Dartford, England).

Analysis of Data

All results are expressed as mean±SEM. The data were analyzed with Student's t-test for paired or unpaired data as applicable. Differences are stated as significant when $p<0.05$ unless indicated otherwise.

RESULTS

Insulin Amylase Ratio

Volume, insulin and amylase concentration and insulin/amylase ratios of freshly processed and cryopreserved grafts are shown (Table IV-1). Cryopreservation causes a significant reduction in the amylase concentration while insulin remains unchanged, resulting in a greater than 3-fold increase in the insulin/amylase ratio.

Portal Pressure

Portal pressure was measured during intraportal embolization of freshly isolated and cryopreserved pancreatic fragments (Figure IV-1). Portal venous pressure increased 16.4±2.3 cm H_2O (mean±SEM) following intraportal infusion of cryopreserved tissue which was significantly less ($p<0.01$) than that seen following the infusion of freshly isolated tissue (31.2±3.3 cm H_2O).

Survival of Dogs (Figure IV-2)

All apancreatic controls became severely diabetic (PG>150 mg/dL) surviving 6.5±0.8 days. Following intraportal embolization of fresh tissue 2 dogs died within 24 h of undetermined cause, while a third dog which was initially normoglycemic (PG=109 mg/dL) died 5 days following engraftment due to intestinal infarction. A fourth dog became progressively diabetic following engraftment and died on the seventh postoperative day. The remaining 2 dogs displayed long-term normoglycemia, one developing hyperglycemia (PG=298 mg/dL) forty days following engraftment and the other remaining normoglycemic throughout follow-up. Intraportal infusion of cryopreserved microfragments was not associated with any acute mortality. All mortality in this group was associated with a return to the diabetic state which occurred early in 3

dogs (day 2, 5 and 14) while 3 dogs remained normoglycemic for an extended period, failing 45 and 75 days after engraftment while the third dog remained normoglycemic permanently. Following renal capsule engraftment of fresh and cryopreserved tissue hyperglycemia rapidly ensued with only 1 dog from either group remaining normoglycemic beyond the fourth post-transplant day. Survival following renal capsule grafts was variable ranging from 1 to 45 days and 5 to 35 days following the engraftment of fresh and cryopreserved tissue respectively.

Autograft Function (Figure IV-3)

Following pancreatectomy control dogs became rapidly hyperglycemic with a plasma glucose (PG) of 428±45 at death. Throughout the 3 month follow-up fasting PG was equal in dogs receiving cryopreserved pancreatic microfragments intraportally to those engrafted with fresh tissue. Recipients of renal capsule grafts of both fresh and cryopreserved tissue became progressively hyperglycemic. The pretransplant K value of all dogs was 3.4±0.2 (n=30). Normoglycemic recipients were re-studied at 1 and 3 months. The K value fell significantly to 1.4±0.4 and 1.0±0.2 ($p<0.01$) 1 month following intraportal infusion of fresh (n=2) and cryopreserved (n=3) microfragments. Three months following engraftment the remaining recipients of fresh (n=1) and cryopreserved (n=1) grafts had K values of 1.5 and 1.3 respectively. Hyperglycemic dogs with renal capsule grafts were not restudied.

Liver Function (Table IV-2)

Alkaline phosphatase rose significantly in recipients of fresh and cryopreserved intraportal grafts ($p<0.01$) as well as fresh ($p<0.05$) and cryopreserved ($p<0.01$) renal capsule grafts within 24 h. Differences

between groups at 24 h were not significant. Serum GGT levels were elevated within 24 h following intraportal embolization of fresh and cryopreserved tissue ($p<0.01$) and after the engraftment of fresh ($p<0.001$) and cryopreserved ($p<0.05$) tissue under the renal capsule. Observed differences between groups at 24 h were not significant. Elevation in LDH occurred following intraportal infusion of fresh tissue ($p<0.01$), within 24 h as well. All these biochemical abnormalities were transient returning to preoperative levels in all cases by 30 days and were not associated with changes in serum bilirubin.

Renal Function

Blood urea nitrogen and serum creatinine measured preoperatively in recipient dogs were 13.6±1.4 and 1.0±0.7 were not affected by transplantation of fresh or cryopreserved tissue to either site.

Coagulation Profile

Coagulation parameters were measured in dogs receiving intraportal grafts (Table IV-3). Prothrombin time and platelet counts were not altered after the infusion of either fresh or cryopreserved tissue. Significant prolongation in PTT occurred 2 h following intraportal embolization of both fresh and cryopreserved microfragments, but had returned to normal in 2 days. Isolated elevation of FDP above 40 mg/L was noted in 1 dog 7 days after intraportal infusion of fresh tissue and 2 h and 7 days following engraftment of cryopreserved tissue in another.

DISCUSSION

Previous studies have suggested that cryopreservation purifies pancreatic microfragments by virtue of the sensitivity of exocrine tissue to cold (11,12). In the present study, purification of canine

pancreatic fragments is demonstrated by a greater than 3-fold increase in the insulin to amylase ratio following cryopreservation. While insulin content remained unchanged cryopreservation reduced the amylase content significantly. These findings suggest that cryopreservation purifies pancreatic microfragments at the expense of contaminating exocrine components and are in keeping with the suggested susceptibility of pancreatic exocrine tissue to injury by the freeze-thaw process (11,12). It has been established that different cell types have different optimal cooling and thawing rates due to their difference in size, membrane permeability and tolerance to osmotic shock (20). The present results suggest that when the cryopreservation protocol is optimized for pancreatic islet cells that selective destruction of contaminating exocrine cells occurs.

Other methods of purifying canine pancreatic microfragments have been reported. Matas et al demonstrated a greater than 6-fold increase in the insulin/amylase ratio of canine pancreatic fragments cultured for 24 h (15,21). During culture they demonstrated a significant fall in amylase concentration. In contrast to the present study in which the insulin concentration was unchanged following cryopreservation they demonstrated a fall in insulin concentration of over 1/2 that of the whole pancreas during culture. When autografted to diabetic dogs the use of cultured islet tissue resulted in a 50% success rate in normalizing PG (21). Nason et al reported the use of high dose radiation to purify canine pancreatic microfragments (22). They demonstrated a 3-fold increase in the insulin/amylase ratio of tissue, following external beam irradiation. They did not demonstrate a significant fall in the amylase concentration of the graft suggesting

that purification occurred by virtue of destruction of non-exocrine tissue contaminating the graft. Following intrasplenic autoimplants Nason and associates were able to reverse diabetes in 70% of dogs followed for 1 month. The induction of islet cell neoplasia, however, is a possible complication of this technique (23). The use of pseudo islet formation by Scharp (24) and Ficoll gradient sedimentation by Alejandro et al (25) has allowed the isolation of highly purified preparations of canine islets. Scharp has been able to successfully reverse diabetes in 25% of dogs transplanted to the spleen with pseudo islets derived from single organs (24). Alejandro and associates have had success transplanting islets purified by Ficoll gradient sedimentation using an allotransplant model, however, insufficient yields of purified islets sometimes necessitated the pooling of purified islets from multiple donors (25).

In the present study in vivo function of grafts purified by cryopreservation was compared to controls of fresh tissue following intraportal embolization and injection under the renal capsule in diabetic dogs. Acute mortality occurred in 3 of 6 dogs following intraportal infusion of fresh graft while the use of cryopreserved pancreatic microfragments was associated with early graft failure in 3 of 6 dogs. This would suggest that intraportal embolization is not the optimal route for engraftment of pancreatic microfragments, when compared to the spleen which has been used reliably for engraftment of both fresh (14) and cryopreserved (10) tissue. Prolonged normoglycemia was achieved, however, in 2 of 6 dogs engrafted with fresh tissue and 3 of 6 treated with cryopreserved tissue. Fasting PG in dogs receiving cryopreserved grafts was equal to that of control dogs engrafted with

fresh tissue throughout the 3 month follow-up. K values 1 month after engraftment were equal as well. This demonstrates that the function of cryopreserved grafts was equal to that of fresh controls following intraportal embolization.

Embolization of unpurified pancreatic tissue to the liver has been associated with a number of severe complications both experimentally and clinically (5,6,7). In the present study portal hypertension was significantly reduced following intraportal embolization of pancreatic microfragments purified by cryopreservation. Vasoactive substances and thrombogenic materials, released from exocrine cells contaminating the graft, have generally been implicated as the cause of portal hypertension under these circumstances (5,26). The significant fall in amylase concentration seen, in the present study, reflects the loss of exocrine tissue in the graft following cryopreservation and resulted in a reduced load of vasoactive and thrombogenic materials being presented to the portal system, explaining the significant fall in portal vein pressure following engraftment. The degree of portal hypertension seen using cryopreserved tissue was never severe enough to induce small bowel infarction as occurred in 1 dog transplanted with fresh tissue.

Acute abnormalities in liver enzymes occurred consistently in dogs receiving intraportal grafts, and were similar whether the dog received fresh or cryopreserved tissue. These changes were transient having returned to preoperative levels by 1 week in most cases and in all dogs by 1 month and were not associated with changes in serum bilirubin. Transient alterations in liver enzymes following intraportal infusion of islet tissue has been described (27,28) and it has been suggested that such changes reflect hepatocellular damage following intraportal

engraftment. However, in the present study similar changes were seen after injection of fresh and cryopreserved pancreatic microfragments under the renal capsule suggesting that the development of transient liver enzyme elevation is not a site specific finding.

The occurrence of severe systemic hypotension following intraportal infusion of fresh pancreatic tissue has been described and is attributed to vasoactive kinins release from exocrine tissue in the graft (29). The greater degree of such contaminants in the fresh graft may explain the two sudden deaths observed following engraftment with fresh pancreatic tissue, as no such deaths were observed following intraportal infusion of cryopreserved tissue.

Disseminated intravascular coagulation (DIC) has also complicated the infusion of pancreatic microfragments into the portal vein (5). This was not observed following the transplantation of either fresh or cryopreserved grafts. In the present study, dogs were systemically heparinized intravenously and as well heparinized saline was used to flush the catheter through which the graft was administered following each aliquot. The use of heparin both systemically and accompanying the graft intraportally, as well as aprotinin the graft preparation has previously been suggested in the management of pancreatic tissue induced DIC (5,29).

Toledo-Peryra has demonstrated the feasibility of the renal subcapsular region as a recipient site for canine pancreatic fragments (29). He successfully reversed diabetes in dogs by allografting mechanically dispersed pancreatic tissue. In comparison Hesse and Sutherland failed to reverse diabetes in 11 dogs autografted with freshly isolated collagenase digested pancreatic fragments (31). In the

present study, we were unable to reverse diabetes in 12 dogs engrafted under the renal capsule with either fresh or cryopreserved tissue as the dogs became progressively hyperglycemic in all cases. These findings support those of Hesse and Sutherland, and we also feel the renal subcapsular region is an inappropriate site for engraftment of pancreatic fragments. The lack of an adequate blood supply to dilute and remove enzymes released from contaminating exocrine cells under the kidney capsule, likely results in autolysis of the pancreatic islets however the loss of endocrine cells transplanted to this site may also be due to ischemia as this is a relatively avascular area. Although cryopreservation purified grafts significantly it did not sufficiently reduce the amount of contaminating exocrine tissue to allow for survival of islets transplanted beneath the renal capsule.

In summary, purification of canine pancreatic microfragments by cryopreservation is demonstrated by a greater than 3-fold rise in the insulin/amylase ratio of pancreatic tissue following cryopreservation. The function of cryopreserved autografts in the liver was similar to that of freshly prepared tissue with the advantage of significantly reduced portal hypertension during intraportal embolization. The failure of all fresh or cryopreserved autografts to reverse diabetes following injection under the renal capsule suggest that unsuitability of this site for implantation of islet containing tissue in dogs.

Table IV-1. Insulin, amylase concentration and insulin/amylase ratios of freshly processed and cryopreserved grafts.

	n	Spun volume (mL)	Insulin content (mU/mg)	Amylase content (IU/mg)	Insulin/Amylase ratio
Fresh	12	17.6±2.0	2.08±0.20	2.24±0.28**	1.0±0.1**
Cryopreserved	12	12.4±1.5	2.33±0.22	0.83±0.10**	3.3±0.5**

mean±SEM

* weight (g)

** Cryopreserved different than fresh $p<0.001$

Table IV-2. Liver function following intraportal and renal capsule implantation of fresh and cryopreserved pancreatic microfragments.

	Pre-op	1 day	7 day	1 month
ALK. PHOS. (IU/L)				
Fresh intraportal	26±9.2 (6)	145±32 (4)	156±68 (3)	38±2.4 (2)
Cryo intraportal	35±8.7 (6)	302±86 (6)	251±64 (6)	46±20 (5)
Fresh renal capsule	53±9.2 (6)	234±65 (5)	275±129 (5)	242±150 (2)
Cryo renal capsule	50±7.5 (6)	248±46 (5)	325±172 (3)	394 (1)
SGOT (IU/L)				
Fresh intraportal	26±2.9	268±57	48±7.2	38±9.5
Cryo intraportal	27±2.3	161±42	55±12	33±2.0
Fresh renal capsule	35±4.3	271±34	83±30	46±12
Cryo renal capsule	23±1.4	115±29	54±14	101
LDH (IU/L)				
Fresh intraportal	142±22	429±77	287±55	61±16
Cryo intraportal	226±50	292±36	263±75	87±30
Fresh renal capsule	172±12	305±131	160±36	190±55
Cryo renal capsule	89±17	160±40	135±27	108
BILI (mg/dL)				
Fresh intraportal	0.37±0.03	1.0±0.34	0.81±0.14	0.38±0.19
Cryo intraportal	0.74±0.08	1.0±0.33	0.74±0.24	0.72±0.20
Fresh renal capsule	0.82±0.14	0.82±0.42	0.6±0.12	0.45±0.13
Cryo renal capsule	0.38±0.08	143±0.07	3.6±2.9	1.2

( ) = number of dogs

Table IV-3. Coagulation profile of dogs receiving intraportal autografts of pancreatic microfragments.

	pre op	2 hours	2 days	7 days
PT (sec)				
Fresh intraportal	9.5±0.2(6)	9.7±0.08(6)	9.5±0.01(3)	9.5±0.03(3)
Cryo intraportal	9.5 (6)	9.7±0.07(6)	9.5 (6)	9.5±0.03(5)
PTT (sec)				
Fresh intraportal	15.2±1.4	24.5±0.5	19.3±1.5	16±3.0
Cryo intraportal	16.8±0.8	21.8±1.9	17.1±0.6	13.8±0.3
PLATELETS ($x10^3$)				
Fresh intraportal	298±14	302±53	255±23	319±27
Cryo intraportal	280±41	263±37	185±89	305±70
FDP (>40 mg/L)*				
Fresh intraportal	0	0	1	0
Cryo intraportal	0	1	0	1

( ) - number of dogs

* - significant fibrinolysis

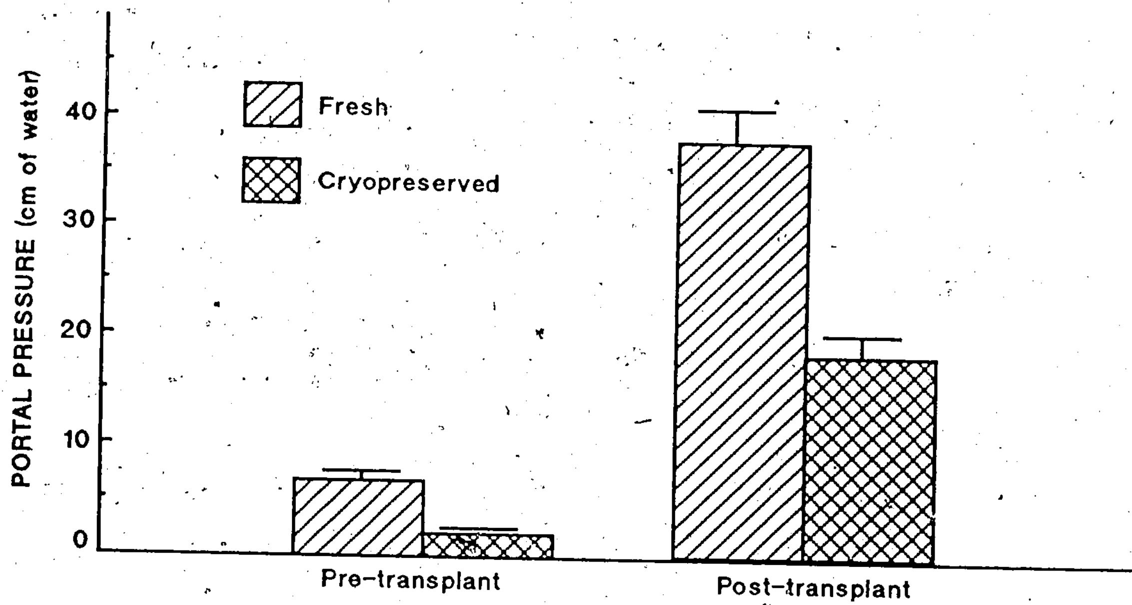

FIGURE IV-1: The effect of intraportal embolization of fresh and cryopreserved pancreatic fragments on portal venous pressure.

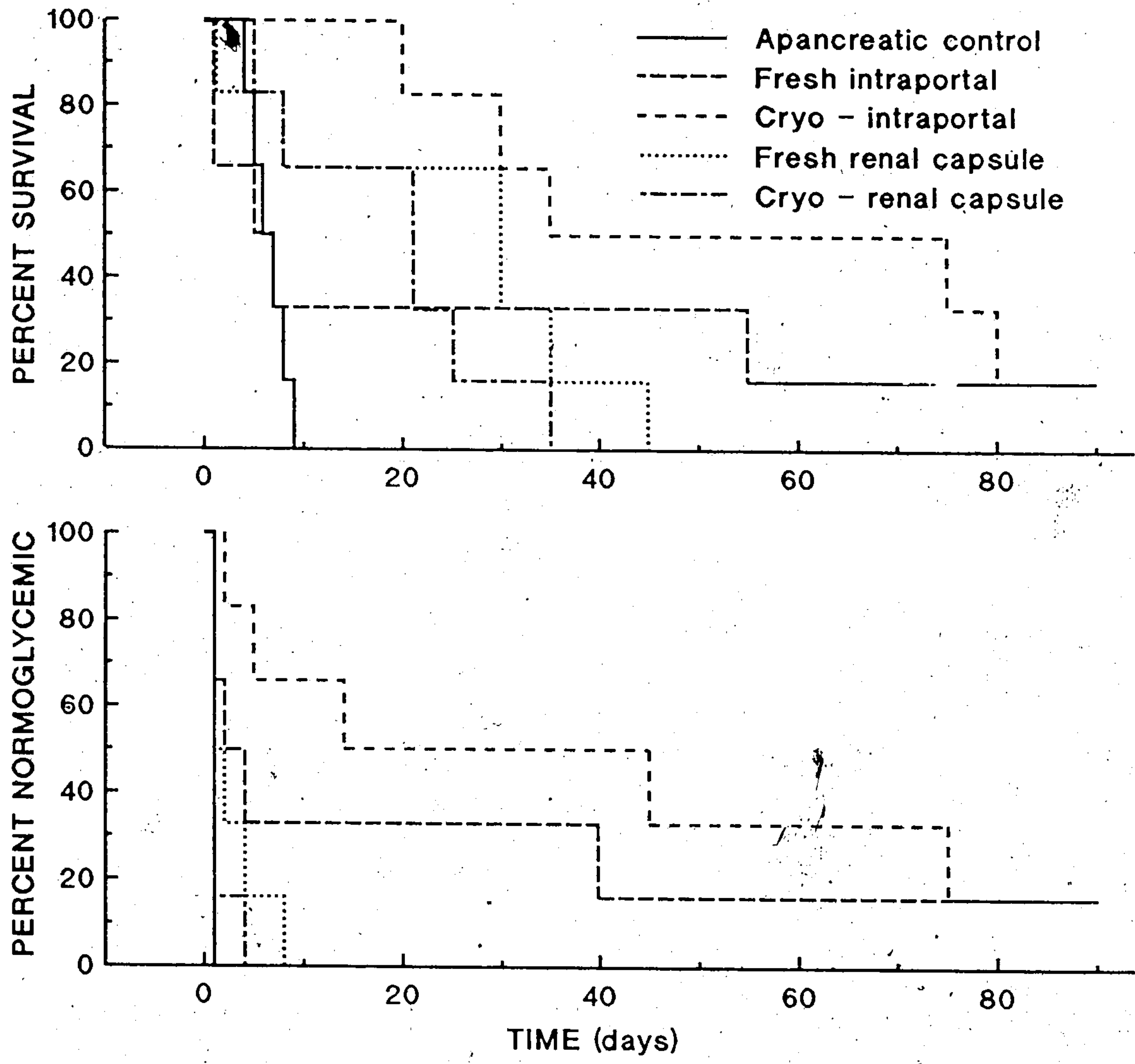

FIGURE IV-2: Survival and normoglycemia of dogs following intraportal and renal capsule engraftment of fresh or cryopreserved pancreatic microfragments.

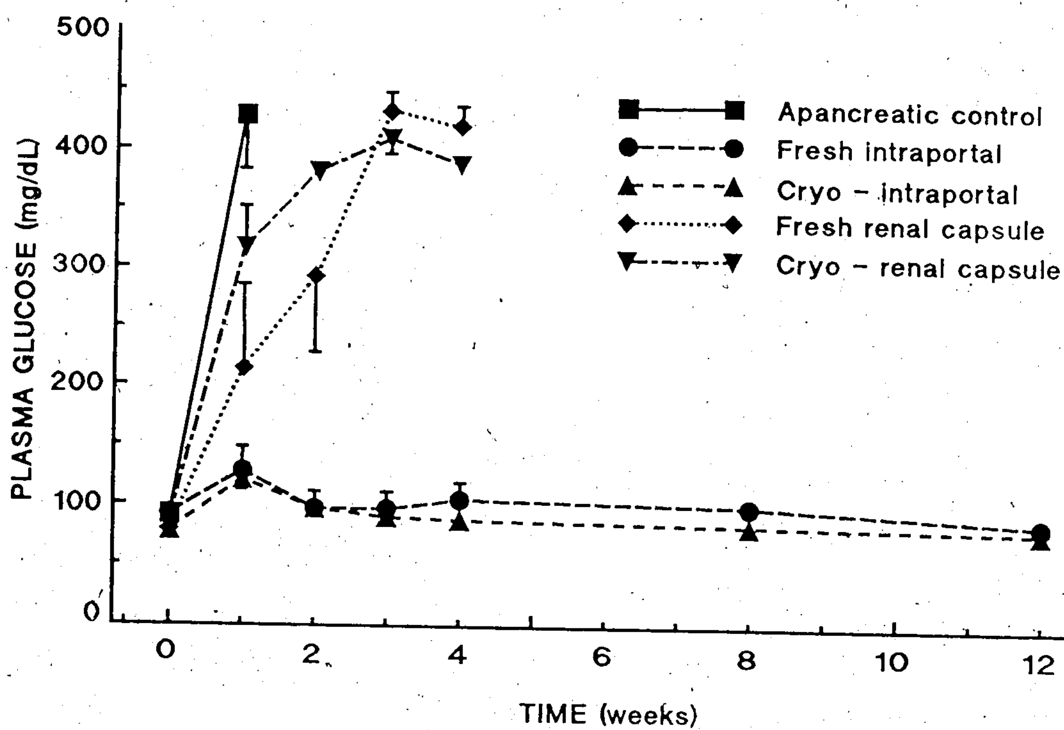

FIGURE IV-3: Fasting plasma glucose of dogs following pancreatic microfragment transplantation. At 1, 2, 3, 4, 8, and 12 weeks n is as follows: Apancreatic 6; fresh intraportal 2, 2, 2, 2, 1, 1; cryo-intraportal 4, 3, 3, 3, 2, 1; fresh renal capsule 5, 5, 4, 4; cryo renal capsule 6, 4, 4, 1.

REFERENCES

1. Mauer MS, Sutherland DER, Steffes MW, Leonard RJ, Najarian JS, Michael AF, Brown DM. Pancreatic islet transplantation effects on the glomerular lesions of experimental diabetes in rats. Diabetes, 1974; 23(9): 748-753.

2. Mauer MS, Steffes MW, Sutherland DER, Najarian JS, Michael AF, Brown DM. Studies of the rate of regression of the glomerular lesions in diabetic rats treated with pancreatic islet transplantation. Diabetes, 1975; 24(3): 280-285.

3. Gray BN, Watkins E. Prevention of vascular complication of diabetes by pancreatic islet transplantation. Arch. Surg., 1976; 111: 254-257.

4. Mirkovitch V, Campiche M. Intrasplenic autotransplantation of canine pancreatic tissue: maintenance of normoglycemia after total pancreatectomy. Eur Surg Res, 1977; 9: 173.

5. Mehigan DG, Bell NR, Zuidema GD, Eggleston JC, Cameron JL. Disseminated intravascular coagulation and portal hypertension following pancreatic islet autotransplantation. Ann Surg 1980; 191 (3): 287.

6. Walsh TJ, Eggleston JC, Cameron JL. Portal hypertension, hepatic infarction and liver failure complicating pancreatic islet autotransplantation. Surgery, 1982; 91: 485.

7. Memsic L, Busuttil RW, Traverso LW. Bleeding esophageal varies and portal vein thrombosis after pancreatic mixed cell autotransplantation. Surgery, 1984; 95 (2): 238.

8. Sutherland DER, Kendal D. Clinical pancreas and islet transplant registry report. Trans Proc, 1985; 17 (1): 307.

9. Rajotte RV, Scharp DW, Downing R, Preston R, Molnar GD, Ballinger WF, Greider MH. Pancreatic islet banking: the transplantation of frozen-thawed rat islets transported between centers. Cryobiol, 1981; 18: 357.

10. Rajotte RV, Warnock GL, Kneteman NM: Cryopreservation of insulin producing tissue in rats and dogs. World J Surg, 1984; 8: 179.

11. Rajotte RV, Warnock GL, Bruch LC, Procyshyn AW. Transplantation of cryopreserved fresh rat islets and canine pancreatic fragments: comparison of cryopreservation protocols. Cryobiol, 1983; 20: 169.

12. H nshaw DB, Jolley WB, Hinshaw DB, Kaiser JE, Hinshaw K. Islet autotransplantation after pancreatectomy for pancreatitis with a new method of islet preparation. Am J Surg, 1981; 142: 118.

13. Cobb LF, Merrel RC. Total pancreatectomy in dogs. J Surg Res, 1984; 37 (3): 235.

14. Warnock GL, Rajotte RV, Procyshyn AW. Normoglycemia after reflux of islet containing fragments into the splenic vascular bed in dogs. Diabetes, 1983; 32 (5): 452-458.

15. Matas AJ, Sutherland DER, Steffes MW, Najarian JS. Short-term culture of adult pancreatic fragments for purification and transplantation of islets of Langerhans. Surgery, 1976; 80(2): 183-191.

16. Liebo SP, Mazur P. Methods in mammalian reproduction. Academic Press, New York, 1979, pg. 179.

17. Morgan CR, Lazarow A. Immunoassay of insulin: two-antibody system. Diabetes, 1983; 12: 115.

18. Sutherland DER, Matas AJ, Steffes MW, Najarian JS. Infant human pancreas: a potential source of islet tissue for transplantation. Diabetes 1976; 25: 1123-28.

19. Moorehouse JA, Grahame GR, Rosen NJ. Relationship between intravenous glucose tolerance and the fasting blood glucose level in healthy and in diabetes subjects. J Clin Endocrinol, 1964; 24: 145.

20. Rajotte RV, Mazur P. Survival of frozen-thawed fetal rat pancreases as functions of the permeation of dimethyl sulfoxide and glycerol, warming rate, and fetal age. Cryobiol, 1981; 18: 17.

21. Matas AJ, Sutherland DER, Kretschmer G, Steffes MW, Najarian JS. Pancreatic tissue culture: depletion of exocrine enzymes and purification of islets for transplantation. Transplant Proc, 1977; 9 (1): 337.

22. Nason RW, Rajotte RV, Procyshyn AW, Pederson JE. Purification of canine pancreatic islet cell grafts with radiation. Transplant Proc, 1986; 18 (1): 174.

23. Sommers SC. In Berdjio CC (Ed). Pathology of Irradiation. Williams and Wilkins, Baltimore, 1971. p434.

24. Scharp D. Isolation of transplantation of islet tissue. World J Surg, 1984; 8: 143.

25. Alejandro R, Cutfield R, Shienvold FL, Latif Z, Mintz D. Successful long-term survival of pancreatic islet allografts in spontaneous or pancreatectomy-induced diabetes in dogs. Cyclosporine-induced immune unresponsiveness. Diabetes, 1985; 34: 825.

26. Traverso WL, Gomez RG. Hemodynamic measurements after administration of aprotinin and/or heparin during pancreatic cell autotransplantation in dog, pig and monkey. Ann Surg, 1982; 195: 479.

27. Kretschmer GJ, Sutherland DER, Matas AJ, Payne WD, Najarian JS. Autotransplantation of pancreatic fragments to the portal vein and spleen of totally pancreatectomized dogs. Ann Surg, 1978; 187 (1): 79.

28. Kolb E, Ruckert R, Largardier F. Intraportal and intrasplenic autotransplantation of pancreatic islets in the dog. Eur Surg Res, 1977; 9: 419.

29. Torres LE, Traverso WL, Yound ZS. Intraoperative hemodynamic changes in patients undergoing mixed-cell L autotransplantation of pancreatic tissue. Anesthesiology, 1980; 53: 427.

30. Toledo-peyera LH, Bandlein KO, Gordon DA, MacKenzie GH, Reyman TA. Renal subcapsular islet cell transplantation. Diabetes, 1984; 33: 910.

31. Hesse UJ, Sutherland DER. Comparison of splenic and renal subcapsular autografting in dogs. Transplantation, 1986; 41 (2): 271.

V

DISCUSSION

Experimental pancreatic islet transplantation has been very successful in both small (1,2,3,4) and large (1,5-9) animals. Since the seminal work by Ballinger and Lacy (2), a number of laboratories have successfully reversed diabetes in rodents by transplanting pancreatic islets (1,3,4). Mirkovitch and Campiche (5) paved the way for large animal studies by demonstrating that by avoiding the steps used for purification, developed in the rodent, sufficient yields of islet containing tissue could be isolated from the more fibrous pancreas of larger mammals to produce normoglycemia following transplantation. Other groups have successfully used this technique to demonstrate the reliability of pancreatic microfragment transplantation for the treatment of diabetes in large animals (6-9,10). It has been shown experimentally that islet cell transplantation not only produces normoglycemia, but that it can also halt and reverse the chronic microvascular changes of diabetes (1,11-19). There are several barriers that must be overcome before clinical islet cell transplantation can become a reality including increasing islet yield, improving graft purity, assuring the optimal recipient site and minimizing allograft rejection (20). The present study, using autotransplantation of islet-containing tissue in the canine model addressed two of these issues. Different sites for engraftment of pancreatic microfragments were compared from the point of view of both function and safety. Graft purification by cryopreservation and its influence on recipient site function was also examined. This study further defines the optimal site

for engraftment in large animals and suggests that cryopreservation is not only the most realistic method for long term preservation of pancreatic tissue, but that it purifies grafts as well.

Comparison of Sites

The ideal recipient site for pancreatic islet containing tissue has yet to be determined. It is generally held that autologous drainage (venous drainage of the graft into the portal circulation) is superior in function when compared to a graft draining systemically (heterologous). This makes sense theoretically since the liver is the organ on which insulin exerts its major action and is supported experimentally by the work of Brown (21) and more recently by Albisser (22). While this concept is widely supported, there have still been a large number of heterologous sites examined in both small and large animals. The superiority of the liver, via portal vein embolization, as a recipient site for pancreatic islets in rodents has been confirmed by several groups since first being demonstrated by Kemp (4). Intraportal embolization, however, has not been as successful in large animals experimentally (7-9) or in limited clinical trials (23,24). The infusion of islet containing tissue into the portal vein has also been associated with a number of unacceptable complications in both dogs and humans, including portal hypertension (8,9,25,26,27), portal vein thrombosis (28), bleeding esophageal varices (28), disseminated intravascular coagulation (25) and systemic hypotension (27,29). For this reason other transplant sites have been examined in the dog model. The spleen first used by Mirkovitch and Campiche has been successfully utilized for the transplantation of pancreatic microfragments. They and others engrafted tissue into the spleen by direct inoculation into the

splenic pulip either by capsular puncture or via catheters advanced through splenic veins (5,6,8,9). Warnock demonstrated improved function by refluxing the graft in retrograde fashion into the spleen from hilar veins (7). The superiority of the spleen for transplantation of pancreatic microfragments in dogs has been demonstrated (9). It appears that the spleen by virtue of its rapid sinusoidal blood flow dilutes thromboplastins and vasoactive amines such that they are presented to the liver in insufficient amounts to cause significant portal hypertension (7).

Until recently, experience with the use of highly purified islets transplanted intraportally had been confined to that of Lorenz (30) who produced normoglycemia for 3 weeks in 10 dogs given intraportal allotransplants of Ficoll isolated pancreatic islets. Alejandro et al (31) have also developed a technique for isolating highly purified dog islets with Ficoll. They successfully reversed diabetes in 16 of 22 Beagles receiving allogenic islets intraportally. In both these studies neither significant portal hypertension or other complications previously associated with portal embolization of pancreatic fragments in large animals occurred.

Heterologous sites have not been investigated in large animals until recently. Toledo-Pereyra et al, successfully used the renal subcapsular region to reverse diabetes in dogs allografted with mechanically dispersed pancreatic fragments (32). In contrast, Hesse and Sutherland were unable to produce normoglycemia in 11 dogs autotransplanted with collagenase digested pancreatic tissue when utilizing the renal subcapsular region (33).

In the present study a comparison between three potential sites was made. Collagenase digested pancreatic microfragments were autografted to the spleen by refluxing through hilar veins, as by Warnock, the liver via portal vein embolization and to the renal subcapsular region. Surgical pancreatectomy was used as a model for diabetes and the consistent development of hyperglycemia and rapid death of all apancreatic controls attest to the severity and reliability of this model. Autopsy failed to reveal any residual pancreatic tissue in these or any other dogs expiring during follow-up. All 10 dogs receiving intrasplenic grafts survived to 1 month, nine being normoglycemic, with graft failure occurring in 1 dog. Although fasting PG (PG) was consistently normoglycemic the fasting plasma insulin was significantly lower at 1 month post-transplant compared to preoperatively (Table A-I). Intravenous glucose tolerance at 1 month was also significantly impaired with a K value of 1.3±0.2% in contrast to 3.5±0.3% prior to pancreatectomy. Peak insulin levels during intravenous glucose tolerance testing (ivGTT) were also reduced significantly (Table A-II).

These findings are consistent with that reported by other authors, supporting the reliability of the spleen as a recipient site for pancreatic microfragments. Kretchmer et al reversed hyperglycemia in 20 of 21 dogs transplanted with pancreatic tissue and followed for 10 weeks (6). Warnock demonstrated similar findings normalizing PG in 9 of 13 dogs autografted to the spleen by splenic vein reflux (7). More recently Hesse and Sutherland autografted pancreatic microfragments to the spleen, by venous reflux as well, and reversed diabetes in 10 of 14 dogs rendered diabetic by total pancreatectomy and followed for 2-6 weeks (33). While normoglycemia is achieved in the fasting state,

impaired glucose tolerance is an invariable finding. In the three previously mentioned studies the mean K value fell from preoperative levels of 3.3±0.27%, 3.4±0.2%, 3.1% to 1.6±0.25 at 2 weeks, 1.4±0.1 at 1 month and 1.47 at 2 weeks, respectively. The reasons for this lack of metabolic efficiency are not totally clear. It has been suggested that islets trapped within fragments of exocrine tissue may not be as effective as free islets, or that the graft by virtue of its bulk cannot be completely supported by the segmental vascular supply of the spleen until neovascularization occurs resulting in further loss of islet tissue (34). Alternatively it may be that the graft contains a marginal amount of insulin secreting tissue which can maintain fasting normoglycemia, but with an inadequate reserve unmasked by glucose challenge. This concept is supported by the significantly lower peak insulin levels during glucose tolerance testing noted in this study and by others (5,6). The value of peripheral vein insulin levels as an accurate parameter of intrasplenic graft secretion is questionable (6,34), since the liver is between the site of transplant and the peripheral veins where insulin levels are sampled. The liver can extract a large portion of the insulin in the portal blood in one pass. Increased hepatic extraction in response to moderate secretion from the graft could result in seriously low peripheral insulin levels. Whether the weakened performance of intrasplenic grafts is a quantitative or qualitative defect is not known, regardless it has been shown that over time the function of intrasplenic autografts as measured by K values improves (35), and stabilizes without evidence of fatigue for up to 18 months (34).

Dutoit documented portal hypertension following inadvertent embolization to the liver of intrasplenic pancreatic tissue (36). Portal pressures were measured before and after injection of the graft. In this study during the infusion of pancreatic microfragments into splenic veins, vascular clamps were placed occluding the hilum of the spleen and left for 10 min following engraftment. There was a slight but insignificant rise in portal pressures following engraftment. Although measurements of this kind following intrasplenic transplantation have not previously been reported in the literature these results are in clear contrast to the degree of portal hypertension well documented to occur during intraportal embolization (8,9). Disseminated intravascular coagulation has been documented secondary to intraportal (25) and intrasplenic (37) engraftment of pancreatic microfragments. For this reason, coagulation parameters consisting of prothrombin time (PT), partial thromboplastin time (PTT), platelet count and fibrin degradation product (FDP) were measured serially following transplantation. A significant drop in the platelet count occurred 2 days following infusion of the graft into the spleen, but no abnormalities involving any other parameter occurred, excluding a consumptive coagulopathy as a potential complication of intrasplenic engraftment.

Liver function was also assessed following transplantation. Significant elevation occurred in the mean Alkaline Phosphatase (Alk. Phos.), serum glutamic oxalacetic transaminase (SGOT) and lactate dehydrogenase (LDH) 24 h post-infusion, but these values had returned to preoperative values by the end of 1 week in most cases and by 1 month in all dogs. Bilirubin was not altered. Similar transient elevations in

liver enzymes following intrasplenic infusion of pancreatic tissue have been reported previously by both Kolb et al (8) and Kretschmer and associates (9). Renal function as assessed by blood urea nitrogen and serum creatinine levels was not altered by intrasplenic transplantation.

In summary, the spleen appears to be an excellent site for the engraftment of pancreatic fragments as demonstrated by a 90% success rate up to 1 month, with metabolic efficiency in this study, equal to that previously reported. The site is safe with no technical complications occurring and no long-term effects on liver, renal or coagulation function.

In contrast to the success of intrasplenic transplants only 2 of 6 dogs receiving intraportal pancreatic microfragments became normoglycemic, these being the only recipients to survive 1 month. One dog died solely due to graft failure and complications were a significant problem with other dogs. One dog died 4 days post-transplant due to complete small bowel infarction and was hyperglycemic at that time. At autopsy this dog did not demonstrate portal vein thrombosis. The small bowel infarcted secondary to severe and persistent portal venous hypertension due to the graft infusion. This dog developed portal venous pressure in excess of 42 cm of water, which was the limit of the manometer, and was probably substantially higher. The graft was infused more rapidly than intended and this may have been responsible for the severity of the portal hypertension. Torres and associates have demonstrated that the incidence of complication from intraportal infusion of pancreatic tissue is related to the rate of injection (29). Two dogs died suddenly within 24 h following engraftment. Documentation of graft function was not achieved

before death and autopsy did not reveal the cause of death in either of these dogs. Specifically they did not have evidence of complications related to portal venous hypertension, or a bleeding disorder. It is possible that hyperinsulinemia resulting in hypoglycemia and hypokalemia was responsible for these sudden deaths. This has been described in dogs receiving whole organ allografts (38). Blood sugars were drawn within 1 h of engraftment in random dogs (not these two specifically) and hypoglycemia was not noted, however, serial glucose and electrolyte determinations during the first 24 h following transplantation were not performed routinely so that definite conc sions can not be drawn. Systemic hypotension following intraportal embolization of dispersed pancreatic tissue has been described (29) and may have led to the demise of these dogs. The possibility of an anesthetic related death, however, cannot be ruled out for these dogs.

The two successful intraportal transplants demonstrated equal fu ction when compared to dogs receiving intrasplenic grafts and had significantly higher fasting insulin levels. Intravenous glucose tolerance testing at 1 month was performed revealing a K value of 1.4±0.04 which compares favorably with that of the intrasplenic autografts. Just as in dogs receiving intrasplenic grafts these dogs have significant glucose intolerance compared with their preoperative state.

Intraportal infusion of canine pancreatic microfragments has previously been reported (7-9,39). Kretchmer and asso es produced normoglycemia in 3 of 10 dogs transplanted with colla se digested pancreatic tissue (9). In contrast to the present study graft failure was responsible for the death of all 7 unsuccessful recipients. Post-op

K values were significantly lower then preoperative values similar to the present study, however, the mean K value following intraportal engraftment was well within the diabetic range (0.77±0.1) in Kretchmer's series. Kolb et al (79) successfully reversed diabetes in 5 of 5 dogs by intraportal embolization of pancreatic fragments, and Horoguchi and Merrell (39) had similar results in 3 of 5 dogs.

Portal hypertension was a consistent finding in all dogs in this group. The volume infused was the same as that transplanted into the spleen suggesting that the elevation in portal pressures seen in this study is specific for intraportal route of pancreatic engraftment. Venous engorgement of the small bowel was seen in proportion to the degree of portal pressure elevation in all dogs, resulting in infarction of small bowel and death in 1 animal. Portal hypertension in varying degrees has been described both in dogs and in clinical trials following the embolization of pancreatic tissue into the portal vein (8,9,25,26,27). It has been attributed to vasoactive substances and thrombogenic materials derived primarily from the exocrine tissue of such grafts. Kretchmer et al (9) documented transient elevation in portal pressure in dogs, but greater elevations have been described leading to severe complications such as portal vein thrombosis (28), bleeding esophageal varices (28) hepatic infarction and death in some patients (26). Coagulation abnormalities have also been demonstrated in conjunction with portal hypertension following portal infusion of pancreatic tissue. Mehigan and associates documented prolonged [illegible] thrombocytopenia and increasing FDP in association with consi[illegible]e perioperative bleeding in dogs and 1 patient (25) following embolization of pancreatic tissue to the liver.

The administration of aprotinin and heparin either in the graft or intravenously has been advocated to manage portal hypertension and coagulapathy (25,40). Heparin should block the consumption of clotting agents in the liver by interfering with high levels of tissue thromboplastins found in autografts. Aprotinin has been suggested because this inhibitor may block proteolytic vasoactive agents and thromboplastins as well (40). Mehigan added Heparin at a concentration of 30 USP units/mL and aprotinin at 200 KIU units/mL, to the graft and showed a significant reduction in portal pressure and improved clotting function in dogs (25). Kolb et al administered 2500 USP units of heparin intravenously prior to transplantation, it is not clear whether aprotinin was used in the graft (8). Kretchmer gave heparin intravenously at a dose of 100 USP units/kg body weight, and did not use aprotinin (9). Traverso studied the effects of heparin and aprotinin following administration of pancreatic shock factor derived from collagenase digested pancreas in dogs, pigs and monkeys (40). Aprotinin in doses of 5000 KIU/kg or 10000 KIU/kg given with the PSF intraportally, blocked the occurrence of portal hypertension in pigs, was only partially effective in dogs and did not work in monkeys. The use of heparin 500 USP unit/kg given intravenously did not effect the severity of portal hypertension above, in any species although it would be expected to prevent secondary thrombosis.

In this study portal hypertension occurred in all dogs transplanted to the liver and was directly responsible for the death of one. Significant coagulation abnormalities were not associated with intraportal infusion, however, aprotinin in a dose of 200 KIU/mL was administered with the graft and heparin was given intravenously at a

dose of 150 USP units/kg. Given the wide variation of dosages for these agents reported in the literature the efficiency, dosage and route of administration of these agents requires further clarification.

Liver enzymes (LDH, Alk. Phos., SGOT) were measured serially and as in the spleen group they all became elevated 24 h following engraftment, and were not associated with significant changes in serum bilirubin levels. The values at 24 h, however, were not significantly different from dogs engrafted in the spleen and similarly returned to preoperative values by 1 month post-transplant. These findings are in agreement with those of Kolb et al (8), but are slightly at variance with those of Kretschmer and associates (9) who found that liver enzymes were minimally elevated following engraftment in the spleen when compared to the degree of elevation seen following intraportal infusion. Although one could presume that these changes reflect hepatocellular damage due to intraportal embolization of pancreatic tissue, the fact that the same changes occurred following intrasplenic injection in this study would argue against such a conclusion.

In summary, when successful engraftment in the liver occurs similar glucose homeostasis as that seen with intrasplenic grafts can be expected, however, with the islet cell preparation used in the present study the development of significant acute complications following intraportal engraftment would indicate the spleen is a superior recipient site.

Transplantation of pancreatic islets to the renal subcapsular region has successfully reversed diabetes in rodents (1), however, until recently this site had not been examined in large animal models. The subcapsular region of the canine kidney was shown to be a potential

recipient site by Toledo-Peryra and associates when they reversed diabetes in pancreatectomized dogs with allografts of pancreatic microfragments mechanically prepared without collagenase digestion (32). Normoglycemia was maintained without immunosuppression or with azathioprine (2.5-5 mg/kg/day) for greater than 6 months suggesting that the renal subcapsular region may also be an immunoprivileged site. In contrast to this study Hesse and Sutherland were unable to produce normoglycemia in 11 dogs autograft with collagenase digested pancreatic microfragments (33). The findings of the present study are in keeping with those of Hesse and Sutherland. None of the 6 dogs with islet tissue engrafted under the renal capsule remained normoglycemic. It routinely took 2 or 3 days for fasting sugars to reach diabetic levels probably reflecting early graft function. All dogs were hyperglycemic by the end of 1 week with fasting glucose values in the range seen with apancreatic controls. Absence of β cell function was substantiated by the fasting plasma insulin values of 1 mU/L routinely, which is the lower limit of sensitivity for the assay (Table A-I). As in the study of Hesse and Sutherland the survival of the dogs was prolonged compared with apancreatic controls (with 4 of 6 dogs surviving 30 days), which again may reflect initial graft function. Another possibility is that marginal function was provided by viable islets that may have implanted in the retroperitoneum after leaking out of the renal subcapsular space. Such small leaks were occasionally observed during engraftment although the bulk of the tissue was placed beneath the renal capsule.

In the present study, islets were not observed in the tissue under the renal capsule when examined histologically, the specimens consisted only of necrotic and fibrotic tissue. These results contrast

dramatically with those of Toledo-Peryra et al. They used a graft prepared solely by mechanical means without the use of collagenase digestion and performed allografts rather than transplanting autologous tissue as in the present study and that of Hesse and Sutherland. The necessity of collagenase digestion for the preparation of a graft that will reliably reverse hyperglycemia has been documented (6). Although differences in experimental protocol may exclude direct comparison with the work of Toledo-Peryra the results of the present study would support the view that the renal subcapsular region is not an appropriate site for the engraftment of pancreatic microfragments.

The spleen has previously been compared directly to the liver (8,9) and the kidney (33) as a recipient site for pancreatic microfragments. Kolb et al (8) reported equal success with both sites, however, Kretschmer and associates (9) demonstrated success using the spleen in 20 of 21 dogs compared to 2 of 10 when engrafted in the liver via portal vein embolization. Hesse and Sutherland produced normoglycemia in 10 of 14 dogs transplanted to the spleen and were unable to reverse hyperglycemia in any of 10 dogs grafted under the kidney capsule (33).

In summary, the spleen possesses properties considered optimal for the successful engraftment of pancreatic islet containing tissue. This organ has a rich vascular supply which is immediately available to the transplanted tissue (providing nourishment and diluting harmful exocrine contaminants) and provides autologous venous drainage to the liver for its hormones. In the current study 9 of 10 dogs transplanted to the spleen by venous reflux became normoglycemic without any morbidity suggesting the splenic vascular bed is the optimal site for collagenase digested pancreatic microfragments. The liver also has an excellent

blood supply and has potential advantages compared to the spleen. Since the major site of insulin utilization is the liver, an intraportal transplant might be expected to function more efficiently and an intraportal transplant would be technically simpler than using the spleen in the clinical situation (34). The liver being a less immunologically active organ than the spleen may theoretically be a superior site for the transplantation of allogeneic tissue as well. In the present study, however, intraportal infusion of graft was associated with success in only 2 of 6 animals and significant complications accompanied its use, indicating the liver is a less desirable recipient site. The renal subcapsular region is a "potential space" and the infusion of pancreatic tissue into this area is difficult. There is no immediate vascular supply to support the tissue until neovascularization occurs, and as well any functioning tissue that might survive would be secreted into the systemic rather than portal circulation. The renal subcapsular region was associated with graft failure in 100% of animals suggesting that it is not an appropriate site for the transplantation of collagenase digested pancreatic microfragments.

Cryopreservation and Graft Purity

Experimental pancreatic islet cell transplantation has been very successful (1). In rodents intraportal embolization of isolated and purified islets reliably reverses diabetes and halts progression of chronic microvascular changes (13,16,17). Low yields of virtually pure islets has not been a problem in rodent studies because of the ability to pool islets from multiple syngeneic donors. Although Lorenz has reported the successful reversal of diabetes in dogs allografted with islets from a single donor purified with Ficoll gradient sedimentation

(30), direct extension of techniques developed in the rodent model have not generally been successful in large animal studies or in clinical trials, because of inadequate yields of purified islets. Mirkovitch and Campiche demonstrated the ability to reverse diabetes in large animals by transplanting collagenase digested pancreatic fragments, consisting of islet cells as well as contaminating exocrine, endothelial, ductal and immune cells (5). This model has been successfully utilized by others to reverse diabetes in large animals (6,7,8). The contaminating components of this type of graft, particularly the exocrine cells, have been responsible for a number of complications including portal hypertension (8,9,25,26,27), portal vein thrombosis (28), systemic hypotension (27,29) and disseminated intravascular coagulation (25) following intraportal infusion in both experimental models and clinically.

Contaminating components of the graft that express class II antigens, the sole stimulators of certain immune responses, have been implicated in the vulnerability of islet cell grafts to allograft rejection (41). Class II antigens are found in high concentration on dendritic cells and are present on ductal, vascular endothelial cells and exocrine cells. It has been demonstrated that class II antigens are either absent on islet cells or occur in relatively low concentration (42). In the rodent model, by treating isolated and purified islets to remove class II antigen, allograft survival can be prolonged without systemic immunosuppression (43,44). A pure preparation of islets is therefore desirable for immunologic reasons as well as the safety of the transplant procedure.

The ability to store viable canine pancreatic fragments by cryopreservation is well established (45,46). The sensitivity of exocrine tissue to low temperature and evidence that cryopreservation selectively destroys contaminating exocrine cells has been documented (47,48). The present study examined the effects of cryopreservation on pancreatic microfragment purity.

Insulin/amylase ratios indicate that cryopreservation results in a greater than 3-fold purification of canine pancreatic microfragments. Insulin and amylase were both significantly reduced by the collagenase digestion process while cryopreservation resulted in a significant fall in the amylase concentration without effecting insulin content. Histological examination of islet cell preparations by Gomoris aldehyde fuchsin staining show preservation of intact islets following cryopreservation, however, exocrine tissue no longer appears viable demonstrating vascularization and loss of cellular integrity. These findings indicate that cryopreservation does purify pancreatic microfragments at the expense of contaminating exocrine components.

These results are in keeping with the suggested susceptibility of pancreatic exocrine tissue to injury by the freeze-thaw process (47,48). It has been established that different cell types and multicellular structures have different optimal cryopreservation protocols, particularly cooling and thawing rates (49). This reflects the difference in size, surface to volume ratio, membrane permeability and tolerance of the cells to osmotic shock and ice crystal formation in these single and multicellular systems. The present results suggest that when the cryopreservation protocol is optimized for pancreatic islet cells that selective destruction of contaminating exocrine cells

occurs. The effect of cryopreservation on other contaminating cell types was not assessed in this study. Should highly antigenic cells, such as passenger leukocytes and dendritic cells be subject to destruction by freezing increased survival could be obtained following transplantation of islets across major histocompatibility barriers (50).

Other methods of purifying canine pancreatic microfragments have been reported. Matas et al demonstrated a greater than 6-fold increase in the insulin/amylase ratio of canine pancreatic fragments cultured for 24 h (10,51). During culture they demonstrated a significant fall in amylase concentration. In contrast to the present study, in which the insulin concentration actually rose following cryopreservation, they demonstrated a fall in insulin concentration of over one half that of the whole pancreas during culture. Nason et al reported the use of high dose radiation to purify canine pancreatic microfragments (52). They demonstrated a 3-fold increase in the insulin/amylase ratio of tissue irradiated compared to the nonirradiated graft. They did not demonstrate a significant fall in the amylase concentration of the graft suggesting that purification occurred by virtue of destruction of nonexocrine tissue contaminating the graft. The potential for radiation to induce islet cell neoplasia (53) may ultimately limit the usefulness of this approach to purifying pancreatic tissue.

The use of pseudo islet formation by Sharp (20) and Ficoll gradient sedimentation by Alejandro (31) have allowed the isolation of highly purified preparations of canine islets, however, with both these techniques marginal islet yield continues to be a problem.

Islet cell grafts purified by a number of techniques have been autotransplanted in the canine model. When autografted to diabetic dogs

the use of cultured islet tissue resulted in a 50% success rate in normalizing PG or reported by Matas et al (51). They used partial pancreatectomy and streptozotocin to induce diabetes, a less reliable model as demonstrated by the fact that one of their control dogs remained normoglycemic and this makes their autograft results questionable. Nason and associates were able to reverse diabetes in 70% of dogs autotransplanted and followed for 1 month (52). The islet tissue was purified by external beam irradiation and transplanted to the spleen. Scharp has been able to successfully reverse diabetes in 25% of dogs transplanted to the spleen with pseudo islets derived from single organs (20). Alejandro and associates have had success transplanting islets purified by Ficoll gradient sedimentation using an allotransplant model. When adequate immunosuppression was achieved with cycloserine A they were able to reverse diabetes in pancreatectomized Beagles in 16 of 17 animals. Insufficient yields, however, necessitated the pooling of purified islets from multiple donors (31).

In the present study in-vivo function of grafts purified by cryopreservation was compared to controls of fresh tissue following intraportal embolization to the liver and injection under the renal capsule in diabetic dogs. A number of questions were examined by this comparison: 1) will cryopreserved grafts function at either of these sites; 2) how will increasing purity of the graft by crypreserving affect function; and 3) will increasing purity following cryopreservation result in fewer transplant related complications.

Diabetes was successfully reversed for a prolonged period in 3 of 6 dogs following intraportal embolization of cryopreserved tissue, while the remaining 3 dogs each demonstrated graft failure (hyperglycemia)

after a variable post-transplant period. Two of six control dogs receiving fresh tissue demonstrated prolonged normoglycemia. Of the remaining 4 dogs engrafted with fresh tissue, 3 succumbed to acute complications of engraftment while the fourth became hyperglycemic after an initial short period of graft function. Comparison of post-transplant fasting PG levels, in those dogs with prolonged function reveals that the function of cryopreserved grafts embolized into the portal vein is equal to that of control dogs for up to 3 months. This is supported by results of ivGTT tests. When challenged with glucose 1 month following engraftment, dogs with cryopreserved grafts responded in similar fashion to control dogs treated with fresh tissue as demonstrated by K values of 1.0±0.2 and 1.4±0.4 for cryopreserved and fresh grafts respectively. Similar results are seen following glucose challenge 3 months after transplantation when the single remaining dog in each group is compared.

Pancreatic tissue purified by cryopreservation successfully reversed diabetes in 50% of dogs following intraportal embolization. This compares favorably with the use of pseudoislets in which successful reversal [illegible]ed in only 25% of animals after intrasplenic engraftment (20). It has also been shown that islets purified by cryopreservation will successfully reverse pancreatectomy induced diabetes in [illegible]00% of animals when engrafted to the spleen rather than the liver ([illegible]4). Grafts purified by cryopreservation do not require pooling from multiple donor in order to reverse diabetes in dogs as is sometimes the case with [illegible]ll purified tissue (31). Cryopreservation permits purification of pancreatic microfragments similar to that seen following 24 h[illegible]sue culture without reducing the insulin content of

the gr (51) and also that of external beam irradiation (52) but does not ca the risk of neoplastic transformation associated with the latter method.

In this study, complications were associated with the intraportal infusion of pancreatic microfragments. Portal hypertension was consistently obs and resulted in portal thrombosis, small bowl infarction, and of one dog following the use of fresh tissue. Grafts purified by cryopreservation resulted in significantly reduced portal pressures after graft infusion and portal thrombosis did not occur in any dogs after the use of purified grafts. Vasoactive substances and thrombogenic materials released from exocrine cells contaminating the graft have generally been implicated as the cause of portal hypertension under these circumstances (25,40). The significant fall in amylase concentration seen reflects the loss of exocrine tissue in the graft following cryopreservation resulting in a reduced load of vasoactive and thrombogenic materials being presented to the portal system explaining the significantly smaller change in portal vein pressure following engraftment.

Sudden death occurred in 2 dogs following the engraftment of fresh tissue. This may have been due to severe systemic hypotension which has been described following intraportal infusion of fresh pancreatic tissue and attributed to vasoactive kinins released from exocrine tissue in the graft (29). Similar acute deaths were not observed in animals transplanted with grafts partially purified of such contaminants by the cryopreservation process.

Although coagulopathies have been described following the intraportal infusion of pancreatic tissue (25) such was not the case in

the current study. There was no significant difference in the minor clotting abnormalities observed following the use of cryopreserved tissue compared to control dogs receiving fresh grafts. Similarly, transient elevations in liver enzymes were noted following intraportal embolization of tissue purified by cryopreservation but they were not significantly different from those seen in control dogs.

Despite the recent enthusiasm for the use of the renal subcapsular region (32), results of the current study would suggest that it is not a suitable recipient site for pancreatic microfragments. Following the engraftment of fresh pancreatic tissue under the renal capsule in 6 dogs, all became progressively hyperglycemic. It was hoped that by reducing the exocrine component of the graft by cryopreservation that engraftment of microfragments under the renal capsule would be more successful and prolonged function demonstrated. This was not the case, however, as all 6 dogs transplanted with cryopreserved tissue behaved as controls, becoming progressively diabetic. These results reflect the relative avascularity of the renal subcapsular space. Without an immediate blood supply to support the pancreatic tissue and to remove enzymes released by exocrine cells, the graft suffers ischemic necrosis as well as autolysis. Cryopreservation did not sufficiently reduce the amount of contaminating exocrine tissue to allow for the survival of islets transplanted beneath the renal capsule.

In summary, purification of canine pancreatic microfragments by cryopreservation is demonstrated by a greater than 3-fold increase in the insulin\amylase ratio compared to fresh tissue. In-vivo function of cryopreserved grafts is considered in light of the three questions put forth at the beginning of this section. First, cryopreserved pancreatic

microfragments will function following intraportal embolization but not after injection under the kidney capsule. Second, when transplanted by intraportal embolization the use of cryopreserved tissue was not associated with an increased rate of success but did exhibit function equal to that of grafts of fresh tissue. Finally, increasing purity by cryopreservation was associated with the occurrence of fewer and less severe complications after intraportal embolization. Increasing purification further may allow for greater success using the intraportal route for engraftment but any hope of successfully employing the renal subcapsular space as a recipient site should remain guarded.

SUMMARY

The spleen has been shown by a number of investigators to be an excellent recipient site for transplanted pancreatic fragments (5-8). Cryopreservation provides an efficient means of storing pancreatic tissue and purifies pancreatic fragments as well. The results of intrasplenic autotransplantation of cryopreserved dispersed pancreatic tissue using the canine model has previously been reported (54). The liver has some theoretical and practical advantages over the spleen as a recipient site clinically, but its use has been associated with a number of severe complications (8,9,25-29). Recently, the renal capsule was demonstrated, in the dog model, to be another potential site (32). In the present study, function and safety following autotransplantation of fresh pancreatic fragments to the spleen, liver and renal capsule were compared; results would suggest the spleen is the optimal recipient site for fresh pancreatic tissue. It was hoped that increased graft purity following cryopreservation would allow for better success using the

intraportal route of engraftment. This was not the case, however, with 3 of 6 dogs demonstrating successful graft function with cryopreserved tissue compared to 2 of 6 with fresh tissue. A significant reduction in portal hypertension and the absence of transplant related complications followed the use of cryopreserved tissue suggesting that increasing graft purity further may yet allow success with the intraportal route of transplantation. This concept is supported by the recent work of Alejandro, et al, who successfully used the intraportal route to infuse a "highly purified canine islets" (31).

It was also felt that using cryopreserved pancreatic tissue might be advantageous when implanting under the renal capsule. The poor results following the use of cryopreserved tissue was in keeping with that seen using fresh tissue, clearly indicating the unsuitability of the renal subcapsular region as a recipient site for canine pancreatic tissue. The conclusions of the present study are as follows:

- Compared to the liver and kidney, the spleen is a superior recipient site for autografts of fresh collagenase dispersed canine pancreatic microfragments.
- The development of acute complications following intraportal embolization makes this a less desirable engraftment site for fresh pancreatic microfragments.
- The kidney is an unsuitable site for implantation of pancreatic microfragments.
- Portal hypertension follows intraportal embolization of pancreatic fragments but is reduced significantly when grafts purified by cryopreservation are used.
- Portal hypertension does not occur after venous reflux of pancreatic fragments to the spleen.
- Coagulation abnormalities do not occur following implantation of pancreatic fragments in the spleen or liver.

- Cryopreservation purifies canine pancreatic microfragments by selectively destroying the contaminating exocrine component.

- Cryopreserved canine pancreatic microfragments can successfully reverse diabetes following intraportal embolization.

REFERENCES

1. Sutherland DER. Pancreas and pancreatic islet cell transplantation. I. Experimental Studies. Diabetologia 20:161-183, 1981.

2. Ballinger WF, Lacy PE. Transplantation of intact pancreatic islets in rats. Surgery 72(2):175-186, 1972.

3. Reckard C, Zeigler M, Barker C. Physiologic and immunologic consequences of transplanting isolated pancreatic islets. Surgery 74(1):91-99, 1973.

4. Kemp CB, Knight MJ, Scharp DW, Ballinger WF, Lacy PE. Effect of transplantation site on the results of pancreatic islet isografts in diabetic rats. Diabetologia 9:486-491, 1973.

5. Mirkovitch V, Campiche M. Intrasplenic autotransplantation of canine pancreatic tissue: maintenance of normoglycemia after total pancreatectomy. Eur Surg Res 9:173-190, 1977.

6. Kretschmer GJ, Sutherland DER, Matas AF, Steffes MW, Najarian JS. The dispersed pancreas: transplantation without islet purification in totally pancreatectomized dogs. Diabetologia 13:495-502, 1977.

7. Warnock GL, Rajotte RV, Procyshyn AW. Normoglycemia after reflux of islet containing fragments into the splenic vascular bed in dogs. Diabetes 32(5):452-458, 1983.

8. Kolb E, Ruckert R, Largardier F. Intraportal and intrasplenic autotransplantation of pancreatic islets in the dog. Eur Surg Res 9:419-426, 1977.

9. Knetschmer GJ, Sutherland DER, Matas AJ, Payne WD, Najarian JS. Autotransplantation of pancreatic islets in the dog. Eur Surg Res 9:419-426, 1977.

10. Matas AJ, Sutherland DER, Steffes MW, Najarian JS. Short-term culture of adult pancreatic fragments for purification and transplantation of islets of Langerhans. Surgery 80(2):183-191, 1976.

11. Reemsta K, Weber CJ. Pancreas and pancreatic islet cell transplantation. In: Davies-Christopher Textbook of Surgery (Sabistan DC, ed.). Philadelphia: WB Saunders Co., 537-542, 1981.

12. Hoffman L, Mandel TE, Carter WM, Koulmanda M, Martin FIR, Campbell DG, McMillan N. A comparison between islet transplantation and parental insulin in the control of diabetes and prevention of renal complications in mice. Metabolism 32(5):451-456, 1983.

13. Gray BN, Watkins B. Prevention of vascular complication of diabetes by pancreatic islet transplant. Arch Surg 111:254-257, 1976.

14. Federlin KF, Bretzel RG. The effects of islet transplantation on complications in experimental diabetes of the rat. World J Surg 8:169-178, 1984.

15. Weil R, Nozawa M, Koss M, Weber C, Reemsta K, McIntosh R. Pancreatic transplantation in diabetic rats: renal function, morphology, ultrastructure and immunohistology. Surgery 78(2):142-148, 1975.

16. Mauer MS, Sutherland DER, Steffes MW, Leonard RJ, Najarian JS, Michael AF, Brown DM. Pancreatic islet transplantation effects on the glomerular lesions of experimental diabetes in rats. Diabetes 23(9):748-753, 1974.

17. Mauer MS, Steffes MW, Sutherland DER, Najarian JS, Michael AF, Brown DM. Studies of the rate of regression of the glomerular lesions in diabetic rats treated with pancreatic islet transplantation. Diabetes 23(3):280-285, 1975.

18. Krupin T, et al. Ocular fluorophotometry in experimental diabetes mellitus: the effect of pancreatic islets isografts. Invest Opthalmol Visual Sci 18:1185, 1979.

19. Nelson J, Lacy P, Hirshberg G. Megacolon and autonomic neuropathy in diabetic rats. J Neuropath Exp Neurol 35:335, 1976.

20. Scharp D. Isolation and transplantation of islet tissue. World J Surg 8:143-151, 1984.

21. Brown J, Mullen Y, Clark W, Molnar G, Heininger D. Importance of hepatic portal circulation for insulin action in streptozotocin-diabetic rats transplanted with foetal pancreas. J Clin Invest 64:1688-1694, 1979.

22. Albisser AM, Nomura M, Greenberg GR, McPhedran NT. Metabolic control in diabetic dogs healed with pancreatic autotransplants and insulin pumps. Diabetes 35:97-100, 1986.

23. Sutherland DER. Pancreas and islet transplant registry data. World J Surg 8:270-275, 1984.

24. Sutherland DER, Kendal D. Clinical pancreas and islet transplant registry report. Trans Proceed 17(1):307, 1985.

25. Mehigan DG, Bell NR, Zuidema GD, Eggleston JC, Cameron JL. Disseminated intravascular coagulation and portal hypertension following pancreatic islet autotransplantation. Ann Surg 191(3):287-293, 1980.

26. Walsh TJ, Eggleston JC, Cameron JL. Portal hypertension, hepatic infarction and liver failure complicating pancreatic islet autotransplantation. Surgery 91:485-489, 1982.

27. Traverso WL, Abou-ZamZam AM, Longmire WP. Human pancreatic cell autotransplantation following total pancreatectomy. Annals of Surgery 193(2):191-195, 1980.

28. Memsic L, Busuttil RW, Traverso LW. Bleeding esophageal varices and portal vein thrombosis after pancreatic mixed cell autotransplantation. Surgery 95(2):238-242, 1984.

29. Torres LE, Traverso WL, Yound ZS. Intraoperative hemodynamic changes in patients undergoing mixed-cell intraportal autotransplantation of pancreatic tissue. Anesthesiology 53:427-429, 1980.

30. Lorenz D, Lippert H, Tietz W, Worm V, Hahn HJ, Dorn A, Koch G, Ziegler M, Rosenbaum KD. Transplantation of isolated islets of Langerhans in diabetic dogs: I. Results after allogenic intraportal islet transplantation. J Surg Res 27:181-192, 1979.

31. Alejandro R, Cutfield R, Shievold FL, Latif Z, Mintz D. Successful long-term survival of pancreatic islet allografts in spontaneous or pancreatectomy-induced diabetes in dogs. Cyclosporine-induced immune unresponsiveness. Diabetes 34:825-828, 1985.

32. Toledo-Peyera LH, Bandlein KO, Gordon DA, MacKenzie GH, Reyman TA. Renal subcapsular islet cell transplantation. Diabetes 33:910-914, 1984.

33. Hesse UJ, Sutherland DER. Comparison of splenic and renal subcapsular islet autografting in dogs. Transplantation 41(2):271-274, 1986.

34. Kneteman NM. Canine pancreatic fragment transplantation: immunosuppression and cryopreservation. (Thesis). University of Alberta, Edmonton, Alberta, 1985, pg. 105 .

35. Rajotte RV, Warnock GL, Kneteman NM. Autotransplantation of fresh and cryopreserved canine islets. XII Congress of the International Diabetes Federation, Madrid, Spain, September 23-28, 1985. Diab Res Clin Prac, Supplement 1, no. 1197, S460, 1985.

36. DuToit DF, Reece-Smith H, McShane P, Denton T, Morris PJ. Intraportal embolization of fragments during intrasplenic pancreatic autotransplantation in dogs. Transplantation 30:389-391, 1980.

37. Miller BHR, Bewich M, Compton FJ, Needham JM, Godwin KL, Winter M. Disseminated intravascular coagulation after dispersed pancreas transplantation in dogs: causative agents. Transplantation 36(3):348-350, 1983.

38. Bewick M, Mundy AR, Eaton B, Watson F. Endocrine function of the heterotopic pancreatic allotransplant in dogs: immediate post-transplant period. Transplantation 31:19-22, 1981.

39. Horaguchi A, Merrel RC. Preparation of viable islet cells from dogs by a new method. Diabetes 30:455-458, 1981.

40. Traverso WL, Gomez RG. Hemodynamic measurements after administration of aprotinin and/or heparin during pancreatic cell autotransplantation in dog, pig and monkey. Ann Surg 195:479-485, 1982.

41. Lafferty KJ, Prowse SJ. Theory and practice of immunoregulation by tissue treatment prior to transplantation. World J Surg 8:187-197, 1984.

42. Shienvold FL, Alejandro R, Mintz DH. Identification of Ia bearing cells in rat, dog, pig and human islets of Langerhans. Transplantation 41(3):364-372, 1986.

43. Faustman DL, Steinman RN, Gebel HM, Hauptfeld V, Davie JM, Lacy PE. Prevention of mouse islet allograft rejection by elimination of intraislet dendritic cells. Trans Proc 17(1):420-422, 1985.

44. Hardy MA, Lau HT, Reemsta K. Prolongation of rat islet allografts with the use of ultraviolet irradiation, without immunosuppression. Trans Proc 16(3):865-869, 1984.

45. Rajotte RV, Scharp DW, Downing R, Preston R, Molnar GD, Ballinger WF, Greider MH. Pancreatic islet banking: the transplantation of frozen-thawed rat islets transported between centers. Cryobiology 18:357, 1981.

46. Bank HL, Davis RF, Emerson D. Cryogenic preservation of isolated rat islets of Langerhans: effect of cooling and warming rates. Diabetologia 16:195, 1979.

47. Rajotte RV, Warnock GL, Bruch LC, Procyshyn AW. Transplantation of cryopreserved and fresh rat islets and canine pancreatic fragments: comparison of cryopreservation protocols. Cryobiology 20:169, 1983.

48. Hinshaw DB, Jolley WB, Hinshaw DB, Kaiser JE, Hinshaw K. Islet autotransplantation after pancreatectomy for pancreatitis with a new method of islet preparation. Am J Surg 142:118-122, 1981.

49. Rajotte RV, Mazur P. Survival of frozen-thawed fetal rat pancreases as functions of the permeation of dimethyl sulfoxide and glycerol, warming rate, and fetal age. Cryobiology 18:17, 1981.

50. Coulombe MG, Warnock GL, Rajotte RV. Prolongation of islet xenograft survival by cryopreservation. Diabetes 36(9):1086, 1987.

51. Matas AJ, Sutherland DER, Kretschmer G, Steffes MW, Najarian JS. Pancreatic tissue culture: depletion of exocrine enzymes and purification of islets for transplantation. Trans Proc 9(1):337-339, 1977.

52. Nason RW, Rajotte RV, Procyshyn AW, Pederson JE. Purification of canine pancreatic islet cell grafts with radiation. Trans Proc 18(1):174-181, 1986.

53. Sommers SC. Effects of ionizing radiation upon endocrine glands. In: Pathology of Irradiation (Berdjio CC, ed.). Baltimore: Williams and Wilkins, pg. 434, 1971.

54. Rajotte RV, Warnock GL, Kneteman NM. Cryopreservation of insulin producing tissue in rats and dogs. World J Surg 8:179-186, 1984.

APPENDIX

Table A-1. Fasting plasma insulin following pancreatic microfragment transplantation*.

	Pre-op (n)	1 month (n)
Intrasplenic	4.6±0.4 (10)	2.6±0.4 (9)***
Intraportal	4.8±0.8 (6)	7.0±1.0 (2)
Renal capsule	3.3±0.7 (6)	1 ** (4)

* of those normoglycemic at 1 month (mU/L)
** of those surviving at 1 month
*** less than pre-op value, $p<0.01$

Table A-2. Peak insulin* during ivGTT of dogs following pancreatic microfragment transplantaiton.

	Pre-op (n)	1 month (n)
Intrasplenic	53±7.0 (6)	7.1±1.5 (9)**
Intraportal	51±7.0 (6)	8±2.9 (2)***
Renal capsule	44±3.0 (6)	- - -

* mU/L
** Less than pre-op $p<0.001$
*** Less than pre-op $p<0.01$