University of Alberta

ENHANCING ENGRAFTMENT OF ISLETS OF LANGERHANS AND OTHER CELLULAR THERAPIES FOR DIABETES

by

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Dedication

This work is dedicated to my grandparents, Gaga (Gladys) and Baba (George) and my sister (Christie): a constant source of inspiration in my life.

Abstract

Islet transplantation is promising treatment for certain patients with type 1 diabetes who experience recurrent episodes of hypoglycemia with unawareness. One opportunity to improve on islet transplant outcomes and potentially broaden the recipient population, is to reduce the early post transplant islet loss. Another opportunity is to develop an alternative source of insulin-producing tissue; thus overcoming the need to rely on organ donation.

Mouse islets are used throughout this thesis and their optimal isolation is a crucial step in many of the experiments contained herein. Histopaque provided the optimal isolation kinetics in addition to being the most cost-effective compound, as compared to dextran, iodixanol and ficoll. Apoptosis is the dominant mechanism by which islets are lost in the early post-transplant period. The caspase inhibitor IDN6556 reduced islet graft apoptosis and enhanced islet survival in both mouse and porcine models of islet transplantation. Cytokines, including TNFalpha and IL-1, contribute to islet toxicity in the early post-transplant period. As such, etanercept (TNFalpha inhibitor) has seen increased use in islet peri-transplant cocktails with minimal pre-clinical support. In our studies, the combination of etanercept, and anakinra (IL-1 receptor antagonist) led to improved islet engraftment as compared to either agent alone. One mechanism for this benefit was a significant reduction in apoptotic cells within the islet graft. We also attempted to enhance engraftment by utilizing resveratrol, a

compound with known anti-oxidant and anti-inflammatory properties. Despite its benefit in other fields, it did not produce a benefit to islet engraftment over a wide dosing range. Stem cells are a promising alternative to cadaveric islet procurement. Herein we have demonstrated the ability to ship human embryonic stem cell-derived pancreatic cells (Cyt49) cells which developed pancreatic endocrine function after transplantation in immunodeficient mice. Although long-term teratoma formation was uncovered, this therapy has potential to overcome the reliance on organ donation and may broaden the diabetic recipient population.

Overall, strategies to overcome early islet loss and the reliance on cadaveric donation of islets are presented here. The use of caspase inhibitors to prevent islet apoptosis is particularly encouraging and should be explored in clinical islet transplantation.

Preface

This thesis entitled “Enhancing Engraftment of Islets of Langerhans and other Cellular Strategies for Diabetes”, is submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Experimental Surgery at the University of Alberta, Department of Surgery. Herein is described strategies to both improve on current protocols in islet transplantation, a therapy for select patients with type 1 diabetes, and to provide an alternative source of insulin-producing cells. This original research has been complimented with two review articles and one book chapter so as to place this research in context and to provide an overall view of islet transplantation. Each chapter entry has either been accepted for publication or has been submitted for publication. In each case, this is stated at the beginning of the chapter.

Chapter 1 introduces the reader to islet transplantation and the significant advances that have been made in the field and is presented in two parts. It is intended to provide an overview of clinical islet transplantation with a focus on the advances made within the last two decades (Published in *Current Medical Literature-Diabetes*). This opening chapter is meant to orient the reader to the gains made in islet transplantation while describing some of the areas for improvement including islet engraftment (Chapters 3-6) and the need for alternative insulin-producing cells (Chapters 7-8).

Mouse models have been employed for the majority of the experiments outlined in this thesis. The isolation of functional and viable mouse islets is crucial for the conduction of islet transplant studies. This is especially true in marginal mass islet transplant experiments in which a reduced number of islets are transplanted and the effect of *in vivo* treatments are studied. We employ a density gradient for our mouse isolation, allowing us to separate islets from the surrounding exocrine tissue. **Chapter 2** (in press in *Islets*) explores the effect of various gradient components on the quality and quantity of isolated mouse islets. The results of this set of experiments are crucial to the marginal mass islet transplant experiments which form the foundation of many of the chapters in this thesis.

The following sections begin to explore **strategies to enhance islet engraftment**. Apoptosis is responsible for a large proportion of early islet loss after transplantation. Using caspase inhibitors to prevent early apoptosis could lead to improved islet survival and enhance clinical outcomes. In **Chapter 3** the caspase inhibitor IDN6556, an orally dosed pan-caspase inhibitor, is tested in a mouse model of islet transplantation. The first part of this chapter (**Chapter 3a**) is intended to provide a brief introduction to cell death and caspase inhibtion. While previously tested caspase inhibitors (zVAD-FMK, EP1013) have proven effective in a similar rodent model, they are limited in their ability to be transferred to the clinic. IDN6556, a drug with high portal vein levels after oral dosing, has already been studied in hepatitis patients and in liver transplantation. This makes it an **enticing candidate for clinical islet transplantation** where islets are infused

into the portal vasculature. This chapter was accepted for publication in the journal *SURGERY.*

In **chapter 4** we further explore the caspase inhibitor IDN6556, this time in a large animal model. Using a miniature swine **total pancreatectomy and islet autotransplantation model** we assess the ability of oral IDN6556 to enhance islet engraftment. Specifically, we assess the first-pass effect or orally-dosed therapy and its effects on islets in the portal vein system. Primary endpoints include the return to euglycemia post-operatively and glucose tolerance one month after transplantation. The clinical implications of this study are tremendous: the ability to preserve the transplanted islet mass could increase the rates of insulin independence with single islet infusions and potentially lower the minimal islet mass necessary for this. Although not directly assessed, the potential to treat two patients with one pancreas would greatly improve the effect of islet transpantation.

A number of islet transplant centers employ anti-inflammatory agents in their islet peri-transplant protocols. Initially infliximab (anti-TNFalpha mAb) and now etanercept (anti-TNFalpha dimeric fusion protein) have been used to enhance outcomes with minimal pre-clinical experimental evidence and a lack of randomized trials. A more recent clinical series (n=3 patients) utilized etanercept in combination with anakinra (IL-1 receptor antagonist). In **Chapter 5** we study the effects of etanercept alone and in combination with anakinra on mouse and

human islet engraftment in mouse models of diabetes. Our purpose is to provide experimental evidence for the use of etanercept with or without anakinra and attempt to elucidate possible mechanisms of benefit.

Chapter 6 begins the second part of this thesis with a focus on stem cells and their potential as a treatment for type 1 diabetes. Donor shortage is one of the limitations in islet transplantation; one which the use of stem cells may overcome. In **Chapter 6** we present an overview of stem calls and their potential role in the treatment of diabetes including progress and limitations. Our lab has developed a collaboration with Viacyte in San Diego, a company which has developed a line of human embryonic stem cells (Cyt49) which they can differentiate into pancreatic endoderm. After transplantation into immunodeficient mice, these cells mature and provide pancreatic endocrine function (insulin release in response to glucose). However, when tested by a second group, these cells failed to provide significant c-peptide/insulin release. In **Chapter 7** we test whether pancreatic endoderm cells derived from Cyt49 can be shipped and transplanted at a remote site (our lab) into immunodeficient mice and provide adequate and reproducible endocrine function. We also plan to allow a cohort of mice to survive to one year in order to assess for teratoma formation, one potential side effect of unregulated stem cell growth.

Chapter 8 serves to consolidate the ideas in this thesis and provides future directions that this research may take.

Acknowledgements

I would like to acknowledge and thank several individuals who have supported me throughout my graduate studies and the preparation of this thesis.

A huge “thank you” to Dr Shapiro. I could not think of a better mentor and role model. His high standards, tireless work ethic and constant availability have provided an invaluable source of motivation that has allowed me to reach this point. I knew I could always contact him at any time and he would always have a way of reassuring me and providing constructive feedback.

Drs Colin Anderson and Lori West contributed valuable discussion and feedback at my committee meetings and candidacy examination. Dr Anderson also provided help in my preparation for course examinations and his laboratory personnel were always available for help and assistance.

Drs Peter Light and James Markmann served as external examiners for my graduate examination, and gave generously of their time to review this thesis and participate on my oral defense examination.

During my research I had an opportunity to visit two different laboratories to gain extra skills and insight. Viacyte (San Diego), our stem cell source, hosted me during my first research year. Thank you to Drs Ed Baetge and Evert Kroon.

During my second year I visited the laboratory of Dr Chuck Brunicardi in Houston, Texas. Very welcoming hosts, Dr Brunicardi and Dr She-he Liu allowed me to learn new techniques and develop new ideas in the field of biochemistry and biology. Dr Brunicardi also provided another example of a successful surgeon scientist, role model and mentor. It is very clear why his surgical residents hold him in such high regard. Dr Robert Arch from Pfizer provided access to the caspase inhibitor IDN6556 and for this I am very grateful. Dr Aducio Thiesen provided his expertise in pathology to assist with the stem cell project.

The department of surgery graduate student office, especially Drs Churchill and Rayat ensured that I remained on track and provided useful insight and advice during my training and during the surgery courses. In addition, they both provided their time as chairs for my examinations.

Both Drs David Williams and Daniel Schiller provided assistance and support as program directors in general surgery. I am certainly appreciative of the effort Dr Williams made to ensure that I was able to start my research degree on time. I know that he went out of his way to hold off the residency schedule so that I could start my research on time. Dr Schiller provided excellent advice during our “fireside” chats and made sure I pushed ahead to finish on time. During my graduate degree, I was fortunate to be accepted into the Clinician Investigator Program (CIP). Dr Bigam, as program director, provided a unique perspective on

my research and I found our meetings a source of inspiration and motivation. Our discussions regarding career paths and career planning were refreshing and very much appreciated. I look forward to working with him over the next three years as a clinical resident. The CIP program itself deserves recognition, we are certainly very lucky at the University of Alberta to have such support.

My labmates provided much needed support and motivation. Drs Christian Toso and Shaheed Merani, having both completed a PhD themselves were a constant source of insight and expertise. Even though they left the lab soon after I arrived, they were always willing to answer questions via e-mail. Dr Allison Maciver joined the lab in the middle of my degree and was helpful both as a friend and a labmate. Her help on the large animal study was crucial to its completion. Dr Tatsuya Kin was also a crucial component of the large animal study. His tireless work ethic and expertise were vital to the study's completion. We could not have completed it without him.

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surgery room; harvesting islets, transplanting islets and singing along to the newest hit song on the radio.

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My work could not have been completed without funding. Alberta Innovates Health Solutions (AIHS), the Alberta Diabetes Institute (ADI) and the Clinician Investigator Program (CIP) provided personal financial support while the Diabetes Research Institute Foundation of Canada (DRIFcan) provided much needed financial support for my projects.

Thanks to Dr Eric Meen for being my friend and a huge supporter during this process. I knew that I could always get a much needed laugh when I phoned him and he always provided a unique perspective and the "voice of reason". Thanks big guy.

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have supported me and provided me the opportunity to see what is really important in life. Thank you very much.

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List of Abbreviations

AIR_{ARG}	Acute Insulin Release in response to Arginine
ANOVA	Analysis of variance
AUC	Area under the curve
CITR	Clinical Islet Transplant Registry
CMRL	Connaught Medical Research Laboratories
Cyt49	A hESC line developed by Cythera (now Viacyte)
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular-signal related kinases
FCS	Fetal Calf Serum
GPAIS	Glucose-Potentiated Arginine-Induced Insulin Secretion
HBSS	Hanks' buffered salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hESC	Human Embryonic Stem Cell
HCL	Hydrochloric acid
H&E	Hematoxylin and Eosin
HTK	Histidine-Tryptophan-Ketoglutarate
IE	Islet Equivalents
IGF	Insulin-like growth factor
IκB	Inhibitor of NK-κB
IKK	Inhibitor of IκB kinase
iNOS	inducible nitric oxide synthase

IPGTT	Intraperitoneal glucose tolerance test
i.p.	intraperitoneal
JNK	c-Jun n-terminal kinase
mTOR	mammalian target of rapamycin
MAPK	Mitogen-activated protein kinase
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
PBS	Phosphate Buffered Saline
SEM	Standard Error of the Mean
STZ	Streptozotocin
TUNEL	Terminal deoxynucleotidyl transferase dUTP Nick End Labeling
TNFalpha	Tumor necrosis factor alpha

Chapter 1: Introduction

Advances in Islet Transplantation

A version of this section was peer reviewed and published in *Current Medical Literature – Diabetes* 2010 27(3) 73-86

Leading Article

Advances in Islet Transplantation

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There have been many advances in islet transplantation since the initial attempt in 1893, when Watson–Williams and Harsant transplanted minced sheep's pancreas into the subcutaneous tissues of a young patient with diabetic ketoacidosis [1]. Although the boy died 3 days later, there was a mild improvement in his glucose control. However, the subsequent discovery of insulin and the production of a near-endless supply pushed islet transplantation into the realm of mere research curiosity for a number of decades.

Interest was renewed in the 1970s when Paul Lacy developed a method for isolating the Islets of Langerhans from the rodent pancreas, with subsequent resolution of hyperglycemia when these islets were transplanted into chemically diabetic rodent recipients [2]. However, isolating islets from the human pancreas proved more difficult. It was not until 1989, when Camillo Ricordi and colleagues developed an automated method for human cadaveric islet isolation, that clinical islet transplantation began to soar [3]. This milestone paved the way for Scharp et al. to demonstrate the first case of insulin independence after islet transplantation in 1990, which lasted up to 1 month post-transplant [4]. Further advances and experimentation culminated in the first series of seven patients with 100% insulin independence, making the so-called Edmonton Protocol the standard for clinical islet transplantation [5]. This study showed that transplantation of an adequate islet mass (>11 000 islet equivalents/kg of recipient weight) using multiple islet donors, a glucocorticoid-free immunosuppression regimen, and avoidance of islet culture prior to transplantation could lead to improved outcomes. This historical publication served to increase interest in the area of islet transplantation and instilled renewed vigor in its research.

Global interest in islet transplantation has remained intact, with many recent advances attempting to improve on all aspects of the islet transplantation process, from islet procurement to the transplantation procedure itself and subsequent post-transplant patient care. Although islet transplantation can be a life-saving procedure for some type 1 diabetes patients, it is not without risks, complications, and drawbacks. Multiple donors, chronic immunosuppression, and the overall cost have led many to question the value of the procedure. Despite these concerns, research continues to advance islet transplantation since it has been shown to drastically improve quality of life in type 1

Introduction

There have been many advances in islet transplantation since the initial attempts in 1893 when Watson-Williams and Harsant transplanted minced sheep's pancreas into the subcutaneous tissues of a young patient with diabetic ketoacidosis[1]. Even though the boy died three days later, there was a mild improvement in his glucose control. The subsequent discovery of insulin many years later and the production of a near-endless supply pushed islet transplantation into the realm of mere research curiosity.

Interest was renewed in the 1970s when Paul Lacy developed a method for isolating the Islets of Langerhans from the rodent pancreas with subsequent resolution of hyperglycemia when these islets were transplanted into chemically diabetic rodent recipients[2]. Isolating islets from the human pancreas however proved much more difficult. It wasn't until 1989, when Camillo Ricordi and colleagues developed an automated method for human cadaveric islet isolation, that clinical islet transplantation began to soar[3]. This milestone paved the way for Scharp et al. to demonstrate the first case of insulin independence after islet transplantation in 1990, which lasted up to one month post transplant[4]. Further advances and experimentation culminated in the first series of seven patients with 100% insulin independence making the so-called "Edmonton Protocol" the standard for clinical islet transplantation[5]. Highlights of this series include transplantation of an adequate islet mass (>11,000 islet equivalents/kg recipient

weight) using multiple islet donors, a glucocorticoid-free immunosuppression regimen and avoidance of islet culture prior to transplantation. This historical publication served to increase interest in the area of islet transplantation and instilled renewed vigor in its research.

The global interest in islet transplantation has remained intact with many recent advances attempting to improve on all aspects of the islet transplantation process from islet procurement to the transplantation procedure itself and the subsequent post-transplant patient care. While islet transplantation can be a life-saving procedure for certain type 1 diabetic patients, it is not without its risks, complications and drawbacks. Multiple donors, chronic immunosuppression and the overall cost have led many to question the value of this procedure. Even still, research continues to advance islet transplantation since it has been shown to drastically improve the quality of life in brittle type 1 diabetic patients. There are many areas of the islet transplantation procedure where significant advances have been made and are still continuing to be discovered. Even though the choice of post-transplant immunosuppression garners much interest, one can not forget the many other areas of the procedure in which positive changes can lead to improved outcomes. Here we review both the historic and more recent advances in islet transplantation and provide a look into the future of this developing procedure (Figure 1-1).

Islet Transplantation: a brief overview (Figure 1-2)

Currently, candidates for islet transplantation include those type 1 diabetics unresponsive to intense insulin therapy and with severe hypoglycemia, especially hypoglycemia unawareness. In addition, patients already on immunosuppression for a renal transplant are ideal. Once a candidate is selected for the program they are placed on a list as for other organ programs. When a deceased organ donor becomes available, the pancreas is harvested and digested to release the islets from the surrounding exocrine tissue. Following further purification, one is left with a reasonably pure preparation containing the insulin-producing islets. At this point, the islets can be transplanted immediately or cultured for further manipulation or to prepare the recipient with immunomodulatory drugs. Islets are infused into the patient's portal vein using a percutaneous technique guided by ultrasound and fluoroscopy. Via gravity infusion the islets enter the portal system to be trapped within the smaller venules within the liver. The tract is sealed to prevent bleeding and the patient is admitted to the hospital ward to be monitored and placed on an intensive glucose management protocol and immunosuppressants. Current outcomes for islet transplantation show >27% insulin independence at three years (CITR registry annual report 2009) with a further 30% of patients on insulin but with positive C-peptide levels. Even more recent discussion has hinted at three year insulin independence rates nearing 45% at five years in certain centers. The goal of even longer-term insulin

independence is possible, but rare, as demonstrated by a recent case report showing >10 year insulin independence[6].

Advances in Islet Procurement and Isolation

Donor Selection

Successful islet isolation begins with proper and thoughtful donor selection. A number of variables are associated with islet isolation outcomes including donor body mass index (BMI), age, cold ischemia time, use of vasopressors and blood glucose levels[7-10]. While a larger pancreas can yield a higher islet mass, it is typically very difficult to predict pancreas size. Kin et al., using data from deceased donors, developed a method for predicting pancreas size and found (i) that pancreas size increases with age (up to the fourth decade), (ii) that males have a larger pancreas than females and that (iii) while BMI correlates with pancreas weight, body surface area (BSA) is a much better predictor[11]. Donors with type 2 diabetes present a problem since their β-cells are reduced in both mass[12] and function[13]. Unfortunately, both the history from the donor family and measurement of blood glucose levels prior to organ retrieval are unreliable in predicting underlying glucose intolerance. As such, it has been suggested to measure HbA_{1c} levels prior to organ decision[14]. Some of the initial scoring systems tried to account for these factors, however, they failed to correlate the scores to post-transplant outcomes; instead focusing on islet isolation success[15, 16]. An improved, standardized system taking into account

both islet isolation and transplantation success would no doubt improve donor selection and transplant outcomes.

Pancreas Digestion

Enzymatic digestion of the pancreas is a critical step in the islet isolation process. The trimmed and sectioned pancreas is digested in the Ricordi chamber, an automated recirculating apparatus developed by Camilo Ricordi and colleagues at the University of Miami[3]. Freedom of islets from the surrounding exocrine tissue is dependent on cleavage of the collagen which surrounds them. Initial enzyme preparations using collagenase alone were ineffective; the presence of non-collagenase impurities was necessary to enhance digestion[17, 18]. Before 1990, a fermentation product of *Clostridium Histolyticum* was used for pancreas digestion[19]. While providing collagenase in addition to non-collagenase activity, it was hampered by lot-to-lot variability which became a major limitation to successful pancreas digestion[20]. In the late 1990's, Roche (Roche Applied Science, Indianapolis, IN) developed an enzyme blend called Liberase HI. This went a long way towards reducing the amount of lot-to-lot variability although it did not eliminate it completely. Unfortunately, in 2007 a statement was issued which detailed the use of a bovine brain component in the manufacturing of Liberase. To eliminate the risk of prion disease transmission, islet labs ceased to use this enzyme blend. Fortunately, Serva (Serva Electrophorsesis GmbH, Heidelberg, Germany) had developed a collagenase designated NB1. This

enzyme mixture was unique in that the user could adjust the ratio of collagenase and non-collagenolytic activity. While initial isolation success with this blend was disappointing, with some minor adaptations groups were able to achieve a high rate of islet isolation success[21-23].

More recently, Roche has developed a mammalian tissue-free version of Liberase (Liberase MTF) while Vitacyte (Vitacyte LLC, Indianapolis IN) has provided a new novel enzyme blend, Vitacyte HA. Both enzymes have already been tested and compared to Serva NB1 where they have displayed comparable efficacy[24, 25].

Islet Purification

After enzymatic dissociation, islets must be purified in order to reduce the volume infused into the portal vein. It is well known that infusions of large amounts of tissue into the portal vein can lead to thrombosis, embolism and even death[26, 27]. Islet purification takes advantage of the different densities between islets and the surrounding exocrine tissue. Using centrifugation, tissue will migrate and settle to a layer corresponding to their own density. The development of the COBE 2991 (COBE Laboratories Inc., Lakewood, CO, USA), originally designed for blood component separation, became one of the key components in obtaining purified islet preps[28]. A large amount of pancreatic tissue can be processed in a closed, sterile environment using a continuous density gradient. This has

allowed for shorter isolation times and improved islet purity. One must also consider that islet purification is a balance of islet purity and islet recovery. The choice of gradient media is variable, but most groups employ either a synthetic polymer of sucrose (Ficoll; Amersham, Uppsala, Sewden) or a derivative of it. A newer gradient incorporates University of Wisconsin solution (UW) with Biocoll (Biochrom, Berlin, Germany). Using this solution, Kin et al. were able to achieve an islet recovery rate of 77%[29]. Others have had success using an Iodixanol-based isolation gradient[30, 31]. In fact this has been shown to enhance islet recovery[30] and lead to increased β-cell survival in culture through a reduction in chemokine/cytokine production[31]. Overall, islet recovery rates range from 50-85%. There is clearly room for improvement.

Islet Culture

The decision to culture islets or to transplant them fresh is a difficult one. The advantages of culturing islets are (1) it allows time for patients living distant from the transplant site to travel and (2) allows for shipment of islets to other sites (3) permits attainment of therapeutic levels of immunosuppression prior to islet infusion in addition to (4) allowing for additional quality control procedures to take place. Of further note, an early rodent study showed a benefit in allograft survival when islets were cultured versus transplanted fresh[32]. A later study supported this data and reaffirmed the belief that culturing of islets likely leads to reduced immunogenicity[33]. Most islet centers use media (eg Connaught Medical

Research Laboratory 1066) supplemented with human serum albumin as the typical culture medium. One of the main drawbacks of islet culture is the potential for islet loss. In fact, a recent retrospective review of 104 human islet preparations showed 10-20% islet loss after 20 hours of culture with 37 (36%) of these preps losing >20%[34]. The biggest consequence of islet loss is that the sample is unusable for clinical transplantation. Factors contributing to islet loss include longer cold ischemia time, lower islet purity and a higher proportion of large islets in the pre-cultured preparation[34]. Of note, the original Edmonton Protocol study avoided islet culture, instead transplanting fresh islets. Most centers now include a period of islet culture, allowing for the creation of regional islet isolations centers which can ship purified islet preps to feeder sites for transplantation[35, 36].

Appropriate donor selection, thorough pancreas digestion and islet isolation have gone a long way towards improving outcomes in islet transplantation. While they may not receive as much research focus as other areas of the procedure, there is no doubt that improvements in this early area of the transplant protocol can lead to improved function of transplanted β-cells.

Advances in Islet Implantation

Although single-donor islet transplantation has been reported and would allow islet transplantation to gain more widespread use[37] most centers require islets from two to three donors. In fact, one of the main reasons for success of the original Edmonton Protocol was the implantation of an adequate mass of islets: >11,000 islet equivalents per kg recipient weight[5]. As described above, the islet implantation procedure has become quite standard across centers yet there have been a number of important advances which have helped to improve outcomes and limit patient morbidity and mortality

Islet Implantation Site

While an alternative site for islet implantation is being actively sought by a number of research groups, the portal site has remained the dominant site in over 90% of islet implants. The isolation process itself subjects islets to damaging ischemic and physical injury, making them susceptible to post-transplant stresses. Therefore, once transplanted, islets require ready access to a robust vascular supply including an ample supply of oxygen and glucose. In addition, islets require a means of sampling representative glucose levels and an ability to deliver insulin to its target tissues. From a surgical standpoint, an ideal site would be one that is accessible through a minimally invasive procedure and affords adequate monitoring of islets after implantation.

The portal site

Early rodent studies showed superiority of the portal/liver site with respect to the number of islets required to reverse diabetes[38]. Unfortunately, there is an eventual loss of islets, even without immune attack[39]. The reasons are multifactorial, although the instant blood mediated inflammatory reaction (IBMIR) likely plays a very significant role in β-cell apoptosis and islet loss[40]. IBMIR is an instantaneous activation of the coagulation and complement activation cascades, resulting in platelet aggregation and infiltration with neutrophils, granulocytes, and monocytes upon transplantation of islets into the vascular system. High expression of tissue factor on the surface of islets is quick to induce the extrinsic pathway of coagulation, and the rapid activation of factor VIIa, Va, Xa, and thrombin.

Although the procedure for clinical islet portal implantation is relatively straightforward, it is not without complications. The Edmonton group reported the complications of bleeding (8%), portal vein thrombosis (3%) and bile leakage (1%) secondary to the implantation procedure[41]. While the initial bleeding events early on were quite serious, they have now been prevented with the use of Avitene® paste to seal and ablate the transhepatic portal catheter tract when the catheter is withdrawn[42]. The risk of thrombosis can be reduced by inclusion of heparin (70 units/kg, unfractionated) in the islet preparation and full heparin anticoagulation post-procedure.

Other potential sites

While the intraportal site has allowed islet transplantation to reach its current state of success, it does have its drawbacks and has driven researchers to search for alternatives (reviewed in [43]). The kidney subcapsular site, while very useful in rodent models, has never shown promise in the clinical setting. It contains a poor blood supply, a relative lack of oxygen and would necessitate an invasive surgical procedure. The pancreas, although tempting due to its high oxygen content and proximity to the portal system, is relatively inaccessible surgically and would be nullified in the case of islet autotransplantation. In addition, it has been hypothesized that priming of cells within local lymph nodes may enhance the autoimmune attack of transplanted islets. The creation of an omental pouch, which would no doubt be a highly vascular site, has shown islet transplant success in both rat and dog models of diabetes[44, 45]. One could also envision a laparoscopic approach to creation of the pouch, thereby reducing the surgical morbidity. The bone marrow has been explored due to its highly vascular nature and relative ease of access. In fact, syngeneic rodent islets transplanted into the os femoris are able to efficiently reverse hyperglycemia and remain functional for at least one year[46]. There was no evidence of disruption of the hematopoietic system or the stability of the recipient bone. Transplantation of islets into a superficial and easily accessible site would facilitate post-transplant monitoring. The forearm musculature as an islet transplant site has been explored by a number of groups and has even been trialed clinically in the setting of islet autotransplantation[47]. More recent evidence has shown that this

site may, in fact, provide revascularization similar to that seen in the native pancreas[48].

Another potentially promising site is the gastric submucosal space (GSMS)[49, 50]. In this site, islets would experience freedom from IBMIR, a rich blood supply and a high oxygen tension. A pre-clinical study showed that islets implanted into the GSMS endoscopically did better than pigs receiving intraportal islets[50]. The former group received less insulin post-procedure and displayed less early islet loss. This has become an exciting possibility for clinical islet transplantation; future research should elucidate the potential for long-term islet survival at this site.

Advances in Immunosuppression

Early immunosuppression

In the early days of islet transplantation, most were performed as combined islet-kidney transplants; initiating immunosuppression was only justified for solid organ transplants. As such, most agents used were those which had efficacy in that setting. Corticosteroids formed the cornerstone of this regimen. While providing very potent imunosuppression, corticosteroids are known to hasten diabetes development. They reduce insulin affinity, down-regulate insulin receptors and impair peripheral glucose uptake[51-53]. In addition, these effects are more

pronounced in the presence of cyclosporine, a calcineurin inhibitor frequently included in the immunosuppression cocktail. Cyclosporine use in an early canine model showed a progressive, reversible, dose-dependent impairment of insulin secretion[54, 55]. A study in renal transplant recipients mirrored this result showing an increased rate of post-transplant diabetes[56]. Of course, avoidance of β-cell stress and damage is paramount in islet transplantation; as such, researchers searched for alternatives.

The Edmonton Protocol

The landmark study from the University of Alberta changed the face of islet transplantation[5]. Corticosteroids were replaced with sirolimus, a mammalian target of rapamycin (mTOR) inhibitor and tacrolimus, a calcineurin inhibitor. Sirolimus, in addition to its anti-proliferative effects, inhibits the response to IL-2 while tacrolimus inhibits the production and release of IL-2. In addition, an inductive course of daclizumab, an IL-2 monoclonal antibody was employed. The rationale for combining sirolimus and tacrolimus stemmed form canine islet allograft studies demonstrating their synergism[57]. This allowed tacrolimus to be included at low doses. The aim of this protocol was to provide potent immunosuppression which prevented both allo- and autoimmunity without toxicity to the transplanted β-cells. In addition to the seven patients in the original Edmonton Protocol series, this protocol has indeed been used elsewhere with remarkable success[58, 59].

More recent studies have shown that this may actually not be the best combination for islets. Islet cell engraftment, regeneration and functional survival may all be deleteriously affected by sirolimus[60-62]. This is likely one of the factors contributing to islet loss over time. In addition, some patients find sirolimus intolerable due to mouth ulcers and neutropenia. Sirolimus has since been shown to worsen renal function, an effect compounded in the presence of tacrolimus[63, 64]. In the setting of a diabetic patient with likely marginal renal function at best, the result can be quite deleterious. While avoiding corticosteroids contributed to the success of the Edmonton Protocol and the 100% insulin independence in the seven patients, for islet transplantation to carry forward, new immunosuppression protocols were required.

Further Advances in Islet Transplant Immunosuppression

Groups are moving away from Edmonton Protocol immunosuppression and finding successful alternatives. The group in Minnesota, led by Dr Bernard Hering, has utilized a combination of antithymocyte globulin (ATG) and etanercept (anti-tumor necrosis factor-α antibody, Enbrel®) for induction therapy with cyclosporine and everolimus for maintenance[65]. Four of their six patients with hypoglycemia unawareness have remained insulin independent at >3 years.

Another group has modified the existing Edmonton Protocol to include etanercept and exenatide (a glucagon-like peptide 1 (GLP-1) analogue) as induction therapy to enhance islet engraftment [66]. Here they were able to achieve insulin independence with fewer islets as compared to a group receiving the original Edmonton Protocol immunosuppression. The idea of facilitating islet engraftment has the potential to reduce the islet mass necessary to induce insulin independence and prolong islet survival. This will be discussed further below.

In a series of eight patients, Posselt at al. utilized efalizumab (anti-LFA-1) in combination with sirolimus or mycophenolate after ATG induction[67]. All eight patients became insulin independent with half of the patients only needing one islet infusion to do so. Unfortunately, while removal of efalizumab from the market limited the long-term follow-up of this study, it provided reassuring evidence that newer immunosuppressive regimens could provide outstanding clinical results.

Currently at the University of Alberta, we are employing potent t-depletional therapy with either thymoglobulin or more recently alemtuzumab (anti-CD52, Campath-1H) induction therapy for first-time transplants and basiliximab for subsequent infusions. Daclizumab is now off the market since expiry of its patent. Maintenance therapy is the better-tolerated combination of mycohenolate mofetil (MMF) and tacrolimus. This has also led to better graft durability at the 3 and 5

year post-transplant points. In addition, we are exploring the use of alemtuzumab induction; once again showing robust graft function at 3 years post-transplant with 75% insulin independence. Essentially, sirolimus has been eliminated from our islet transplant protocols, as this agent, at least at high dose, has been associated with the majority of clinical side-effects after islet transplantation, and may also interfere with islet engraftment and regeneration.

Future Advances

Overcoming Donor Shortage

Donor shortage is a problem in islet transplantation as in any other organ transplant program. However, since current protocols call for multiple donors to meet and maintain insulin independence in most centers, this shortage is an even bigger dilemma. Furthermore, as islet transplantation becomes applicable to a broader range of patients and as the incidence of diabetes increases this problem will be compounded.

Living Donor Islet Transplantation

One possibility to address the donor shortage is to use living donors. Living donors have been utilized in both kidney and liver transplantation with great success. In the case of islet transplantation, a distal pancreatectomy would be performed on the donor and the islets isolated. Presumably, these islets would

be harvested under ideal conditions which would augment some of the variability inherent in islet isolation. There would be minimal cold ischemia and no exposure to inotropic agents meaning that these islets should have superior function as compared to cadaveric islets. Furthermore, there would be much closer HLA matching between donor and recipient, assuming that they are related. Of course a downside of this procedure would be if the donor developed diabetes. In the analogous setting of the pancreas living donor undergoing hemipancreatectomy, Kendall et al. showed an impairment of insulin secretion and glucose tolerance one year after the procedure[68]. Whether these donors develop clinical diabetes mellitus remains to be seen.

There have been three cases of living donor islet transplantation to date in the literature[69, 70]. Two of these occurred in 1978, many years prior to the Edmonton Protocol. While neither recipient had received sustained islet function, this pioneering effort was quite remarkable given the early stage of islet transplantation. There has been only one further attempt since the development of the Edmonton Protocol. This occurred at the University of Kyoto in 2005 employing sirolimus and tacrolimus immunosuppression with infliximab (anti-TNF-α) induction. The recipient, a 27 year old with C-peptide negative diabetes secondary to chronic pancreatitis, not autoimmune diabetes, had remained insulin independent for at least the first two months following transplantation[69]. Her mother (the donor) had not shown any sign of glucose intolerance. It is difficult to draw conclusions from a single successful case. Of note, the islet

mass infused was only 8,200 IE/kg which is directly in line with islet autotransplantation data from Minnesota showing > 75% insulin independence with islet preps >2500 IE/kg[71, 72]. In these cases, pancreatectomy is performed for chronic pancreatitis. One further point to draw from these data is that in a setting free of auto- and alloimmune islet attack, graft function is stable over time. This points towards the immune system as the main reason for the loss in islet mass and insulin independence over time.

Xenotransplantation

Developing an alternative source to human islets is obviously an attractive option; especially if this source were renewable. Pigs have become a promising source for islets since they are readily available and produce insulin which is functional in humans. Several small clinical trials have been conducted using porcine islets but unfortunately there have been very few cases of long-term insulin independence[73-75] although there has been promising success using non-human primates as recipients[76, 77]. Of particular note is the work from Emory University led by Dr Larsen. Using either neonatal porcine [78] or adult porcine[79] islets in a diabetic monkey model they have shown both islet engraftment and insulin independence when co-stimulation blockade immunosuppression is used. Unfortunately, there is still concern over transmission of zoonotic infections from pigs to humans. That being said, this is a promising area of research with further clinical studies underway[80].

Stem cell Transplantation

Another potential source of insulin-producing cells is from stem cells. Stem cells, capable of proliferation and self-renewal, can be derived from a number of different tissues and in the case of β-cells could be derived from embryos, bone marrow, the liver or even the pancreas itself (reviewed in [81]). However, there has always been an intense ethical debate surrounding their use; especially when an embryonic source is considered.

Some exciting advances have been reported utilizing umbilical cord blood (UCB). An easily obtainable source with the potential to avoid some of the ethical conflict, UCB was initially tested in animal models showing no evidence of endogenous β-cell replenishment[82]. Surprisingly enough though, two clinical studies have shown reduced insulin requirements[83] and indeed insulin independence[84] in newly diagnosed type 1 diabetics receiving autologous UCB.

Embryonic stem cells (ESCs) have been a focus for many researchers due to their pluripotency or the ability to differentiate into many different tissues given the proper signals. Determining an effective set of “signals” to allow ESCs to become insulin-producing cells has not been easy. Kroon and colleagues in California were successful in developing glucose-sensitive, insulin-producing

cells which could ameliorate diabetes in a rodent model[85]. One caveat of this type of cell is the potential to form teratomas secondary to their growth and differentiation potential. Even so, this type of research could provide an endless supply of insulin-producing cells. A further type of explored cell population is the induced pluripotent stem cell (iPS). These are cells which can be dedifferentiated to a more pluripotent state before differentiation. These have the attractive possibility of originating from the patients own cell population, and avoiding the use of immunosuppressive therapy. A recent mouse study has shown the ability of these cells to reverse hyperglycemia[86].

Preventing Islet Loss After Transplantation (Figure 1-3)

From the moment islets are implanted in the portal system there is loss of this precious insulin-secreting tissue. Up to 60% of this loss can take place in the first few days after transplantation while the islet are trying to find a foundation in their new environment. However, there is a continued, steady loss over time secondary to both allo- and autoimmune attack.

Improved Islet Engraftment

Facilitating islet engraftment during the first few days following islet implantation could lead to reduced initial islet loss (up to 60% can be lost initially[87]). Fewer lost islets would mean that lower islet masses could be transplanted which in turn would open up more marginal islet preparations for transplantation. Islet isolation disrupts the microvascular environment of islets which can take up to two weeks

to redevelop in the host[88, 89]. Other factors including hypoxia, hyperglycemia, the immunosuppressive cocktail and IBMIR all contribute to this early islet loss.

In brief, a number of strategies have been employed to prevent early islet loss including prevention of IBMIR[90, 91] which has led to heparin use in the post-transplant period[92]. In other studies, prevention of apoptosis, the main route of islet death, has been shown to enhance diabetes reversal in mice after marginal mass islet transplantation[93, 94]. It is likely that even more approaches to prevent islet loss will surface in the near future.

Newer Immunosuppressive Agents

As mentioned above, immunosuppression in islet transplantation has seen significant changes since the early days of corticosteroid use. More effective and less toxic drugs have supplanted even the original Edmonton Protocol immunosuppressive regimen. A search of clinicaltrials.gov shows that there are a number of new drug trials actively recruiting subjects(Table 1-1). In addition to true immunosuppressive agents, groups are searching for compounds to promote islet survival and function.

In 2004, the National Institutes of Health (NIH) created the Clinical Islet Transplant Consortium (CIT). This provides a means for islet centers to conduct large multicenter trials with the goal of improving islet transplant outcomes. This

collaborative group involves some of the biggest islet transplant centers in the world including Atlanta, Chicago, Edmonton, Minnesota, Miami, Norway, Philadelphia, San Francisco and Sweden. This group alone is spearheading a number of innovative islet transplant studies, the outcomes of which will continue to advance the field of islet transplantation and immunosuppression (Table 1-1, denoted with *).

Conclusions

The field of islet cell transplantation has advanced immensely since its early roots. Changes in islet procurement, isolation, transplantation and post-transplant immunosuppression have all led to the results we are seeing today. Insulin independence and, more importantly, improvements in quality of life for type 1 diabetics are a reality after islet transplantation. The future of this experimental field is promising and will likely produce even further advances in the years to come.

Figure 1-1 Advances in Clinical Islet Transplantation.

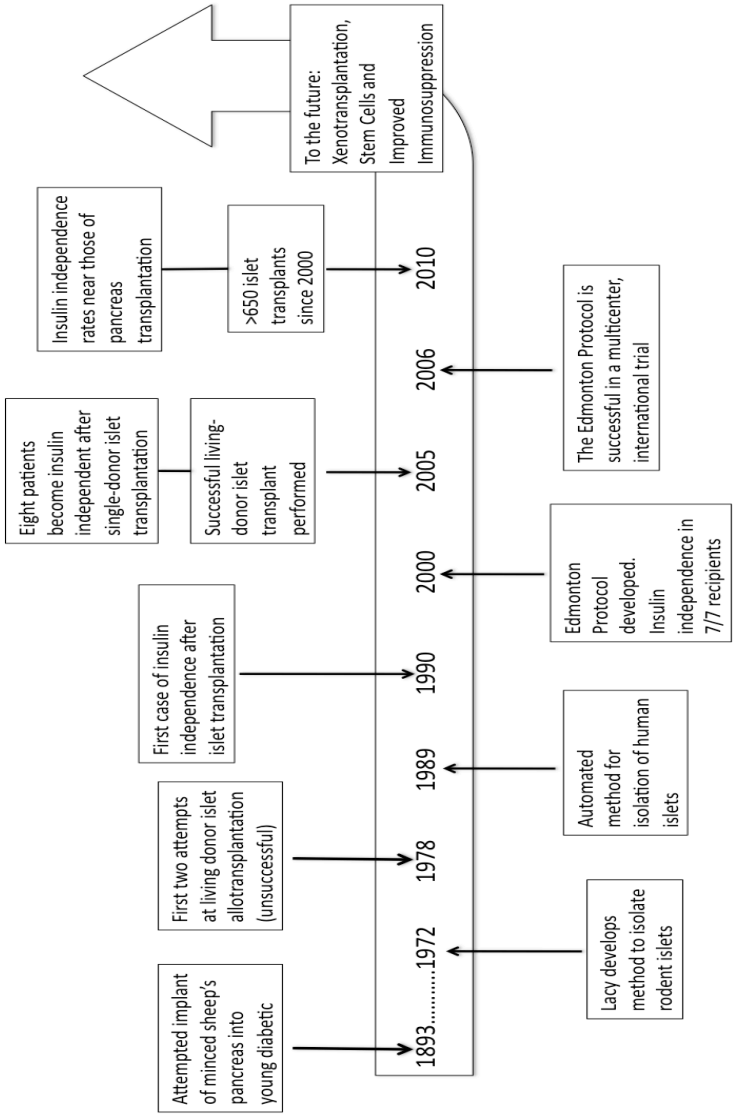
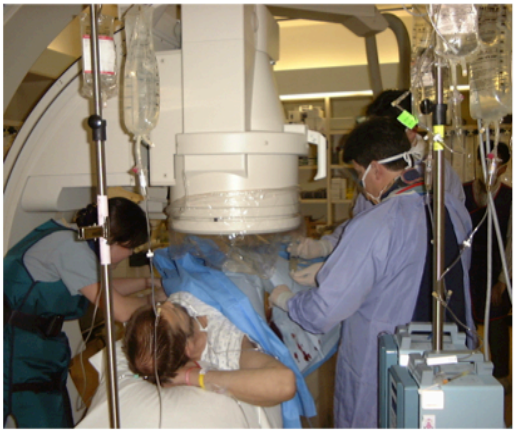


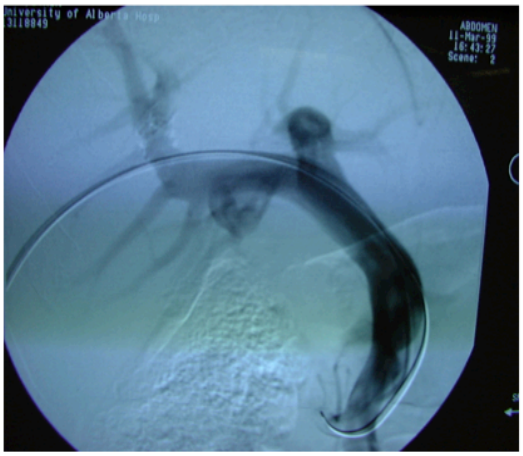
Figure 1-2 The islet implantation procedure.

(A) Islet transplantation requires a coordinated team effort (B) Infusion of purified islets (C) Portal Angiography



a)

b)



c)

Figure 1-3 Preventing islet loss

There are multiple reasons for both early and late islet loss.

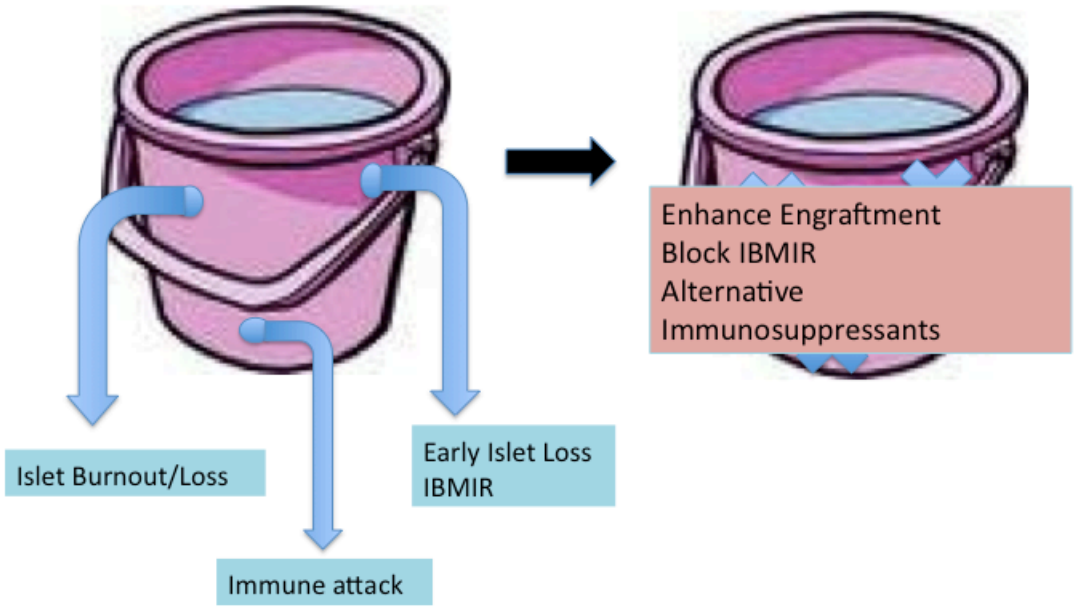


Table 1-1 A summary of currently recruiting clinical trials in islet transplantation.

Centre	Drugs being trialed	Phase
Edmonton (U of Alberta)	Alemtuzumab/Tacrolimus/MMF	III
*Edmonton (U of Alberta)/Atlanta (Emory)	Belatacept (LEA-29Y)	II
*CIT-07 – All participating centers	Daclizumab/Sirolimus/Tacrolimus/Etanercept	II
Miami	Alemtuzumab/Sirolimus/Tacrolimus/MMF	II
*Miami/U of Illinois (Chicago)	Lisofylline (anti-infalmmatory)	II
*Norway/Sweden	Low Molecular Weight Dextran Sulphate	II
Wisconsin (University of Madison)	Thiazolidinedione (a glitazone)	II
*UCSF/Northwestern(Chicago)/ Minnesota	Deoxyspeguelin (anti-inflammatory)	II
U of Illinois (Chicago)	Basiliximab/Tacrolimus/Siroimus/Etanercept/Exenetide	III
Vancouver (UBC)	Sitagliptin (a DPP-IV inhibitor)	III
* CIT-06 (All CIT Centers)	Islet after kidney transplant (IAK)	III

Included were trials exploring new immunosuppressive agents or combinations.

(MMF=Mycophenylate Mofetil, DPP=Dipeptidyl Peptidase). *Denotes a Clinical

Islet Transplant Consortium Trial (CIT)

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Overview of Islet Engraftment

While islet transplantation has become a promising treatment for select type 1 diabetic patients, there is still room for improvement. One of the main shortcomings of the procedure is the **lack of long-term insulin production**. This is equated to a reduction in the percentage of patients remaining insulin independent in the years following islet transplantation. A **loss of islet mass is likely the main contributor to this problem**; both in the early post-transplant period (up to 60% of the transplanted mass can be lost) and in the years following. **Finding ways of reducing the early islet loss may improve long-term outcomes**. Looking at this another way, a reduction in the initial islet loss would reduce the minimal islet mass necessary for a successful transplant.

Islet engraftment, the incorporation of the graft into the host, is a crucial time period in islet transplantation. Developing means to enhance engraftment should lead to enhanced islet survival and function. **We explore ways to improve engraftment in animal models** including the islet isolation gradient (Chapter 2) the caspase inhibitor IDN6556 (Chapters 3 and 4), potent anti-inflammatory agents (Chapter 5) and an anti-oxidant/anti-inflammatory agent (Chapter 6).

Chapter 2

Preface to Chapter 2

The majority of the studies in this thesis revolve around the use of mouse islets and their transplantation. The purification of mouse islets involves collagenase digestion of distended pancreata with subsequent islet purification. One method of purification employs a density gradient; taking advantage of the differential density of islets and the surrounding exocrine tissue. While Ficoll was historically the constituent of choice, issues with subsequent islet viability arose andalternatives sought. Early on in my research, our lab was introduced to Histopaque, a Ficoll-based product. While cheaper and easier to use (pre-made, no mixing required) we were unsure of its effect on islet purification outcomes. At about the same time, iodixanol was introduced to the islet world as providing optimum results for human islet isolation. As such we asked the questions:

1) Is Histopaque a **viable alternative** for mouse islet isolation?
2) Does Iodixanol provide **optimum isolation kinetics for mouse islets** (since it has proven useful in clinical islet isolation)?
3) How does **cost and preparation time** factor into this decision?
4) Since the majority of my studies revolve around mouse islets, **does the choice of islet purification gradient compound affect islet engraftment and function?**

This chapter focuses on these questions. **In fact, results from these experiments have changed practice in our lab.**

Histopaque provides optimal mouse islet purification – comparison study with Ficoll, Iodixanol and Dextran

A version of this chapter was peer-reviewed and accepted to the journal *Islets* (in press)

RESEARCH PAPER

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Histopaque provides optimal mouse islet purification kinetics

Comparison study with ficoll, iodixanol and dextran

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Key words: islets of langerhans, gradient, histopaque, ficoll, islet isolation

Abbreviations: HBSS, Hank's buffered salt solution; ELISA, enzyme-linked immunosorbent assay; RPMI, Roswell Park Memorial Institute

Islet transplantation has become a very promising treatment for type 1 diabetes. To facilitate further clinical improvements in this exciting field, rodent islets are used to evaluate new strategies and modifications. One method to purify islets is on a density gradient, although the optimal gradient component can be debated. N = 6 separate mouse islet isolations were used and the resulting islets were separated and purified on either a Ficoll, Histopaque, Dextran or Iodixanol gradient. Islets were assessed for recovery, viability, purity and in vitro functionality. Aliquots were transplanted into diabetic mice to assess in vivo functionality and survival. There was no difference in the number of islets recovered across groups nor in the size of recovered islets. Use of a Ficoll or histopaque gradient led to the most pure and viable islets in comparison to dextran and iodixanol. Functionally, islets isolated on a Ficoll gradient had the highest glucose-stimulated insulin release in vitro while performing equally to histopaque and dextran gradients in vivo. Using a Ficoll gradient, however, comes at a higher monetary cost. We recommend using a Histopaque gradient, which led to the isolation of viable and functional islets with a reduced cost as compared to a Ficoll gradient.

Introduction

Diabetes is a large worldwide problem, causing an estimated 5% of deaths worldwide (WHO, World Health Organization). This number will likely increase by 50% in the next 10 years without urgent action. For highly selected patients with unstable and intractable hypoglycemic control and type 1 diabetes, islet transplantation has become a viable and life changing option. The Edmonton Protocol,[1] a follow-up international trial[2] and now extensive international experience in more than 700 cases with further refined protocols, demonstrated the possibility of insulin independence even though there is concern over declining graft function over time.[3] However, for islet transplantation to see more widespread use and to allow longer periods of insulin independence, further research needs to be conducted.

While performing studies on isolated human islets is crucial, the availability of human donors can be a roadblock. Rodent islets serve as a useful surrogate for human islets and provide an inexpensive and reliable means of studying islet transplantation. Since the first description in 1967 by Lacy and colleagues[4] rodent islet isolation has become a relatively straightforward and reproducible means of isolating islets. Even still, it is advantageous to develop an optimal isolation protocol that minimizes cost and animal usage while at the same time providing purified, functioning and healthy islets.

The process of rodent islet isolation consists of three main steps: collagenase distension and perfusion, pancreas digestion and islet purification. Islets can be purified by varied means including culture[5] filtration[6] and gradient separation. A number of compounds have been proposed as the optimal gradient constituent including Ficoll,[4,7,8] Ficoll-based Histopaque,[9] Dextran[10,11] and a newer compound Iodixanol.[12,13] Each of these solutes has its own osmolality, differences in which can lead to varied islet numbers, viability, purity and functionality. Each of these factors can affect the way in which islets behave once transplanted; an important consideration for both allogeneic and syngeneic transplant models. In addition,some have questioned the use of Ficoll due to its potential toxicity to islets which can affect both islet yield and functionality.[6,12,13] Not to be overlooked are financial considerations; is it justifiable to use a more expensive gradient for only a marginal benefit to outcomes? In this study we aim to provide a critical and comprehensive comparison of four currently employed mouse islet purification gradients.

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The process of rodent islet isolation consists of three main steps: collagenase distension and perfusion, pancreas digestion and islet purification. Islets can be purified by varied means including culture (5) filtration (6) and gradient separation. A number of compounds have been proposed as the optimal gradient constituent including Ficoll (4, 7, 8), Ficoll-based Histopaque (9), Dextran (10, 11) and a newer compound Iodixanol (12, 13) (Figure 2-1, Table 2-1). Each has its own osmolality, differences which can lead to varied islet numbers, viability, purity and functionality. Each of these factors can affect the way in which islets behave once transplanted, an important consideration for both allogeneic and syngeneic transplant models. In addition, some have questioned the use of Ficoll due to its potential toxicity to islets which can affect both islet yield and functionality (6, 12, 13). Not to be overlooked are financial considerations: is it justifiable to use a more expensive gradient for only a marginal benefit to outcomes?

Purpose: In this study we aimed to provide a critical and comprehensive comparison of four currently employed mouse islet purification gradients.

Materials and Methods

Animals and Reagents

BALB/c mice were obtained from the Jackson Laboratories and housed under conventional conditions. All animals were cared for according to the guidelines of the Canadian Council on Animal Care, and ethical approval was obtained from the animal welfare committee at the University of Alberta. All reagents were obtained from Sigma Aldrich unless otherwise specified.

Mouse Islet Isolation

BALB/c mice were anesthetized using 1% isoflurane and a midline laparotomy was performed. The common bile duct was cannulated with a 27 guage needle and the pancreas distended with 3 cc of cold HBSS (Hanks' buffered salt solution) with 1 mg/ml collagenase. Pancreata were removed and the mice euthanized. Collagenase digestion was carried out at 37°C with collagenase (1mg/ml in HBSS). Following 15 minutes of digestion, the solution was strained to separate the undigested tissue.

Islet Gradient Isolation

After pancreas digestion and separation, the resulting tissue was separated into four equal groups and islets further isolated on one of four gradients (Table 1). Tissue was resuspended in 12.5cc of the first layer of the gradient in 50cc conical tubes after which 6cc of the following layers were slowly added. HBSS constituted the final layer. All gradient solutions were stored at 4°C; Ficoll and Histopaque gradients were allowed to reach room temperature before use. Gradients were centrifuged at 2000 RPM for 10 minutes after which the islets were washed twice with 20cc of HBSS.

Ficoll gradient: To prepare Ficoll stock (25%), Ficoll powder was dissolved in HBSS to a final density of of 1.1g/cm^3 a final pH of 7.2. This stock solution is pressure-filtered using a 0.22μm filter. Further dilutions of the stock were created using HBSS to 21.5 and 11.5%.

Dextran gradient: Dextran stock (25%) was prepared at 0.33 g/ml in EuroCollins solution with 0.79 g/L benzamidine (EC-Benz) and adjusted to a final density of 1.1g/cm^3, ph 7.2. Subsequent dilutions of 21.5% and 11.5% were created using EC-Benz.

Histopaque: Histopaque stock (Histopaque-1088) was diluted using HBSS to create dilutions of 21.5% and 11.5%.

Iodixanol: OptiPrep™ stock (Axis-Shield) was diluted in HBSS to a final density of 1.1 g/cm^3. Further dilutions were created using HBSS to 21.5 and 11.5%.

Islet Counting, Viability, Insulin and Protein Content and Purity

Purified islets from each of the gradients were hand-counted under light microscopy. Three aliquots of one hundred (100) islets from each purification group were handpicked and viability was assessed using Syto green/ethidium bromide (Cedarlane laboratories) using established methods (*24*). Three independent observers calculated the viability on the three separate samples and the results were averaged. These results were then averaged over the islet isolations. Two hundred and fifty (250) islets were collected in triplicate for analysis of insulin content. Insulin extraction was performed in acid-ethanol by homogenization and ultrasonic cell membrane disruption. Insulin concentration of the neutralized extract was measured using a commercial ELISA kit specific for mouse insulin (Alpco Diagnostics). Protein content was determined on five hundred (500) islets using a Bradford assay.

Glucose-stimulated insulin release assays

For each gradient, duplicate aliquots containing 100 islets were washed three times in RPMI-1640 media (Roswell Park Memorial Institute-1640 media with 2.5% Penicillin/Streptomycin and 5g/L bovine serum albumin). The islets were then moved to 6 ml low-glucose (RPMI-1640 as above plus 0.56 mg/ml D-glucose) for 60min followed by high-glucose medium (RPMI-1640 media as above containing 4.4 mg/ml D-glucose) for a further 60min. Each incubation was carried out at 37°C, 5% CO2. Aliquots from the supernatants were analyzed in triplicate for mouse insulin content using an ELISA kit (Alpco diagnostics). In each experimental condition, the stimulation index was calculated by dividing the mean insulin released from islets cultured in high-glucose medium by the mean insulin released from islets cultured in low-glucose medium.

Islet Transplantation

Streptozotocin was administered to recipient BALB/c mice to render them diabetic (220 mg/kg intraperitoneal). Diabetes was confirmed with two blood glucose readings ≥ 20mmol/L using a OneTouch Ultra glucometer (Lifescan Canada). Mice were placed in surgical plane using 1% isoflurane and a left flank incision was made to expose the kidney. Five hundred spin-pelleted islets were transplanted under the left kidney capsule under sterile conditions. Blood glucose

readings were obtained daily from a tail-vein sample until mice reached euglycemia, considered to be two readings ≤ 11 mmol/L.

Graft Insulin Content

Four weeks after transplantation, euglycemic mice were euthanized and graft-bearing kidneys collected and stored at -80°C until bulk analysis could be performed. Extraction was performed in acid-ethanol by homogenization and ultrasonic cell membrane disruption. Insulin concentration of the neutralized extract was measured using a commercial ELISA kit (Alpco Diagnostics).

Statistical Analysis

Data was analyzed using GraphPad Prism (Version 5.0c, GraphPad Software Inc). P values less than 0.05 were considered statistically significant. Graphical representation of data is shown as mean ± SEM, unless otherwise indicated in the figure legends. Repeated measures ANOVA was used to assess for differences across groups followed by Bonferroni's multiple comparisons test, unless otherwise stated.

Cost Analysis

To determine the cost of each gradient, the cost of reagents, filters and disposables was totaled in $USD (Table 2-1). These costs exclude any personnel wages.

Results

Islet Characteristics

Using N=6 individual isolations of thirty (30) mice each, pancreata were collected and the digested tissue split into four equal groups to be further purified on one of four gradients. After gradient purification, the islet yield was counted for each group and averaged over the six isolations. There was no significant difference in the number of islets obtained per donor pancreas among the groups nor was there a difference in the number of islet equivalents collected (Figure 2-2A and B, $p>0.05$). In addition, the islet ratio was calculated, giving an estimate of islet size (Figure 2-2C). Once again, the isolation gradient itself had no effect on the size of isolated islets ($p>0.05$). Next, we assessed the purity of the islets isolated. Using an ELISA assay, after islet ultrasonication, we determined the concentration of insulin in a sample of 250 unpurified islets and averaged this over N=4 purifications (Figure 2-3A). There was a significantly higher amount of insulin present in the tissue purified using an Iodixanol gradient (5.75 ug/ml, $p<0.05$ versus Ficoll and dextran) as there was using a Histopaque gradient (4.628 ug/ml, $p<0.05$ versus Ficoll). Using a Bradford assay (a spectrophotometric measurement using Coomassie Brilliant Blue) on 500 islets we determined the protein content of the isolated tissue (Figure 2-3B). Once again, using an Iodixanol gradient led to a significantly higher protein concentration in the isolated tissue (135.4 ug/ml, $p<0.05$ versus other gradients). We used the proportion of the protein content made up by insulin as a surrogate

for the purity of the islet isolation (Figure 2-3C). The absolute purity values calculated this way will be much lower compared to the standard method using a ratio of islet tissue to non-islet tissue, however this should still provide a means of assessing the relative purity of each isolation method. Here there was a significant advantage to using a Histopaque or Ficoll gradient (*Histopaque*: 16.2% purity, $p< 0.05$ versus iodixanol and dextran; *Ficoll*: 14.7% purity, $p<0.05$ versus dextran). Aliquots of 100 islets were analyzed for islet viability using a combination of SYTO green and ethidium bromide, assessing for membrane integrity (Figure 2-4). Use of a Ficoll gradient led to viable islets (88.6% viability, $p<0.05$ versus dextran and Iodixanol) although they were not significantly more viable than using a Histopaque gradient (82.0% viability, $p<0.05$ versus Iodixanol).

Islet Functionality

In vitro

We used the same N=6 islet isolations to assess the *in vitro* functionality of isolated islets. Triple aliquots of one hundred (100) islets were collected for each gradient and subjected to low and high glucose media. The resulting insulin release was measured, averaged across replicates and isolations and compared (Figure 2-5A). Isolation on an iodixanol gradient led to islets with the highest amount of insulin release in low-glucose media (16.60 ng/ml, $p<0.05$ versus ficoll). There was no difference among the groups when the islets were subjected

to high-glucose media. Calculation of the stimulation index, an assessment of islet functionality and insulin release potential, showed a significant functional benefit if islets were isolated using a Ficoll gradient (16.64, $p<0.0\text{-}5$ versus all other gradients) (Figure 2-5B).

In vivo

To assess the in vivo functionality of gradient-isolated islets, transplantation into diabetic syngeneic recipient mice was carried out (Figure 2-6). After streptozotocin-induced diabetes was confirmed, recipient mice were transplanted with five hundred islets from one of the gradient purification methods. Ficoll (N=8), Dextran (N=6) and Histopaque (N=7) isolation all led to islets which allowed 100% diabetes reversal of diabetic recipient mice, however only the Ficoll-isolated islets reached statistical significance over Iodixanol (N=9)-isolated islets (73.3%, $p<0.01$) (Figure 2-6A). In addition, Ficoll-isolated islets caused mice to become euglycemic a day earlier (day 1) than dextran-isolated islets (day 2) and two days earlier than both histopaque and iodixanol-isolated islets (day 3). Thirty days after transplantation, mice displaying prolonged euglycemia were euthanized and their grafts collected for insulin content (Figure 2-6B). In these euglycemic mice there was no difference in the graft insulin content across groups ($p>0.05$).

Cost Analysis

The cost of preparing one litre of each of the gradients was calculated and compared (in USD) (Table 2-2). Preparation of a Ficoll gradient incurs the most cost ($950/litre). Both histopaque and iodixanol (Optiprep™) are purchased as pre-prepared liquids.

Discussion

In this study we aimed to compare mouse islet gradient components in order to optimize our mouse islet isolation protocol. Clearly the dynamics of islet isolation and purification of mouse islets are distinctly different from human islet isolation, and advances found in mouse isolation are unlikely to translate to techniques for human isolation. However, mouse islet isolation techniques remain important for future developments that may one day be applied clinically. Determination of the ideal mouse islet gradient solution must take into account a number of factors including islet recovery, viability, purity and functionality. Additionally, one must consider cost and ease of preparation as key factors in this determination. A gradient component which only provides marginal functional advantage but which incurs a larger cost would make a poor choice.

While there is no advantage of one gradient over another in terms of the number of islets recovered, Ficoll and Histopaque gradients have the advantage in terms of islet viability and purity. Less viable islets would likely be more prone to early post-transplant loss through allorejection. In this case, islet damage could release signals which alert antigen presenting cells causing subsequent alloreactive T-cell priming and activation through the “danger model” proposed by Matzinger(*14, 15*). Purity also has important bearing on immune activation, with higher exocrine and lymphocyte contamination more likely to trigger activation events. It is possible that immunological tolerance would be easier to induce in

mouse islet transplantation with a more pure islet preparation. Clinically, islet purity is an important characteristic as impure islets infused into the portal vein can lead to a number of devastating side effects including thrombosis and embolism(*16, 17*). This is likely no different in a rodent model where the portal vein site is used to model the clinical setting(*18*). However, a less pure islet preparation has the advantage of containing ductal and stem cells which could improve long-term clinical outcomes.

Islet functionality is another important consideration during islet isolation and purification. Ficoll-isolated islets showed improved functionality both *in vitro* and *in vivo* although there was no statistical difference as compared to Histopaque in terms of syngeneic diabetes reversal. While Iodixanol-isolated islets released a significantly higher amount of insulin in the low-glucose media, this was likely due to islet death as these islets also had the lowest viability. Although Ficoll-isolated islets reversed diabetes more quickly in a syngeneic model, this difference did not lead to an improvement in overall islet survival as there was no difference between the groups when considering the surviving islet mass.

Ficoll or a Ficoll-derived gradient (histopaque) did not negatively affect islet yield or viability as has been previously reported(*6, 12, 13, 19, 20*). In fact, Ficoll-based gradients led to significantly improved *in vitro* function as compared to dextran and iodixanol gradients. We are careful about limiting the time that islets spend in Ficoll solutions and ensure adequate washing immediately afterwards.

We used a non-marginal mass transplant setting to assess *in vivo* function while others have opted to use a marginal mass approach (6). However, based on the enhanced *in vitro* function of islets isolated on a Ficoll gradient, we would expect these islets to behave similarly in a marginal mass setting.

A number of studies have shown Iodixanol to be an effective gradient composition for the isolation of human (*21*), rat (12) and porcine (*22*) islets. In fact, these studies have shown an iodixanol gradient to enhance islet recovery (*12, 21, 22*) in addition to islet viability (*22*), purity (*12, 22*) functionality(12) and anti-proinflammatory effects(*23*) compared to Ficoll. This last study, performed by Mita et al. showed the efficacy of OptiPrep™ in purifying human islets. When compared to Ficoll, there was no difference in islet purity, postpurification IEQ, islet recovery rate or β-cell viability(*23*). In agreement with this study, we have demonstrated that OptiPrep™ purification yields an increased insulin content of islets yet we failed to demonstrate an advantage in terms of islet viability and *in vivo* function. In the current paper, islets isolated on an OptiPrep™ gradient were relatively impure; the contaminating exocrine tissue likely hindered the engraftment of islets when transplanted into diabetic mice. We were further unable to show a benefit to using an Iodixanol-based islet purification gradient in mouse islet purification. We chose to use OptiPrep™ as our source of iodixanol, following the procedure of van der Burg et al.(*22*). However, we modified that protocol to mainain consistency between gradients, using HBSS as the dilution solution. We also made an attempt to employ gradient layers of similar density

across the gradient groups. This may have led to the suboptimal viability and purity of islets purified on the iodixanol gradient secondary to inadequate removal of pancreatic acinar tissue. It is also possible that iodixanol is ineffective in purifying mouse islets although Delle et al. were able to effectively use Visipaque (Roche) to purify rat islets(12). As far as we know, there is no report of using iodixanol to purify mouse islets. Iodixanol has been found to provide excellent puritication of human islets, based on prior studies (*21, 23*).

Cost and preparatory time are two important considerations when choosing an islet purification gradient. Larger expenditures in both may not be justified for only marginal benefits to islet recovery or function. Here we have shown that both Ficoll and the Ficoll-based Histopaque solution can purify viable and functional islets. While Ficoll purification may give an *in vitro* functional advantage to purified islets, this did not translate into an *in vivo* advantage in terms of function or islet survival. Moreover, this *in vitro* advantage comes at the downside of almost three times the cost and extra preparatory time. Histopaque is purchased pre-mixed and filtered and only needs diluting to the required densities. This considerably simplifies the preparation time. On the other hand, ficoll is purchased as a powder and requires mixing and filtration. We are now employing Histopaque as our mouse islet gradient solution.

Table 2-1 Comparison of gradient compound characteristics

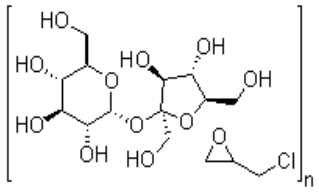
Component	Description	Notes	Other Uses
Dextran	Complex branched glucan	• Synthesized form sucrose by bacteria	• Intravenous volume expansion • Prevent thrombosis
Ficoll	Neutral, highly branched, hydrophilic polymer of sucrose	• Large (>400,000kDa)	• Blood component separation
Histopaque	Combination of polysaccharide (Ficoll) and sodium diatrizoate	• Stock solution produced at 1.077g/ml	• Blood component separation
Iodixanol	Nonionic, iso-osmolar, hexa-iodinated	• Iso-osmolar with blood (290 mOsm/kg) • Free of endotoxin • Lower viscosity than Ficoll	• Visipaque™ used as a contrast agent

Table 2-2. Comparison of gradient composition and cost

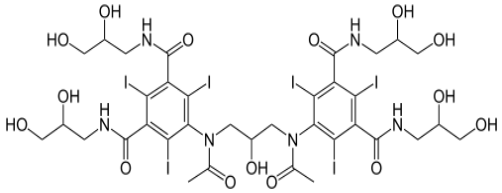
Gradient	Density of Stock (g/ml)	pH	Cost of preparation of 1 litre[a]
Dextran	1.1	7.42	$250
Ficoll	1.1	7.41	$970
Histopaque	1.08	8.45	$330
Iodixanol	1.1	7.46	$215

[a]Excluding salary/wage of person preparing. Includes cost of reagents and any filters required (in approx $USD)

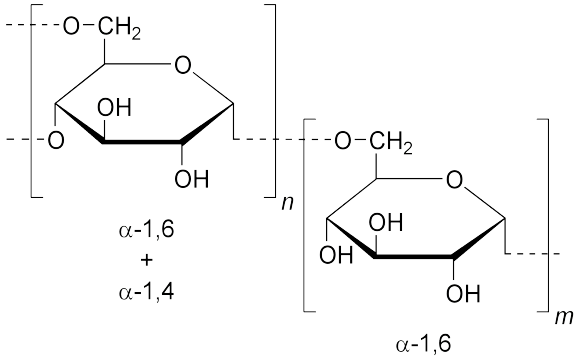
Figure 2-1 Chemical structure of gradient components



Ficoll



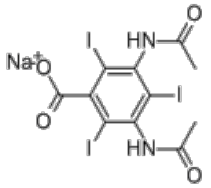
Iodixanol



α-1,6
+
α-1,4

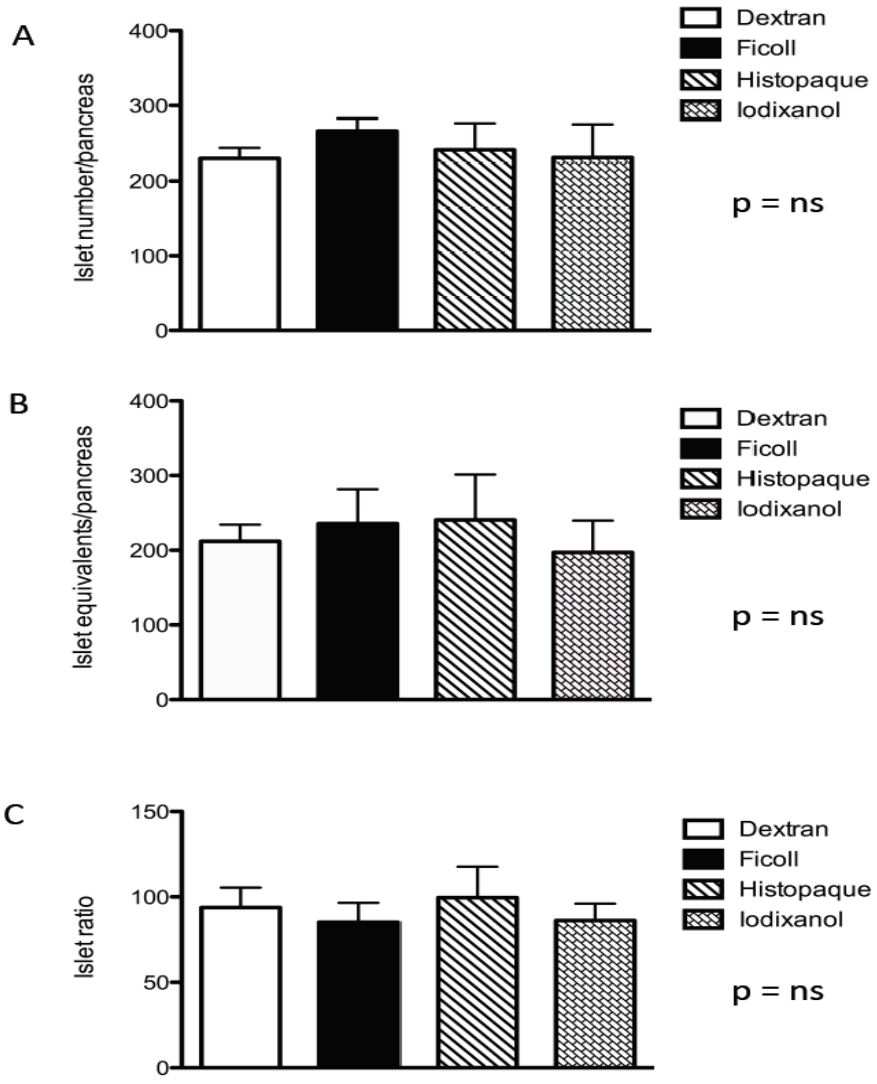
α-1,6

Dextran



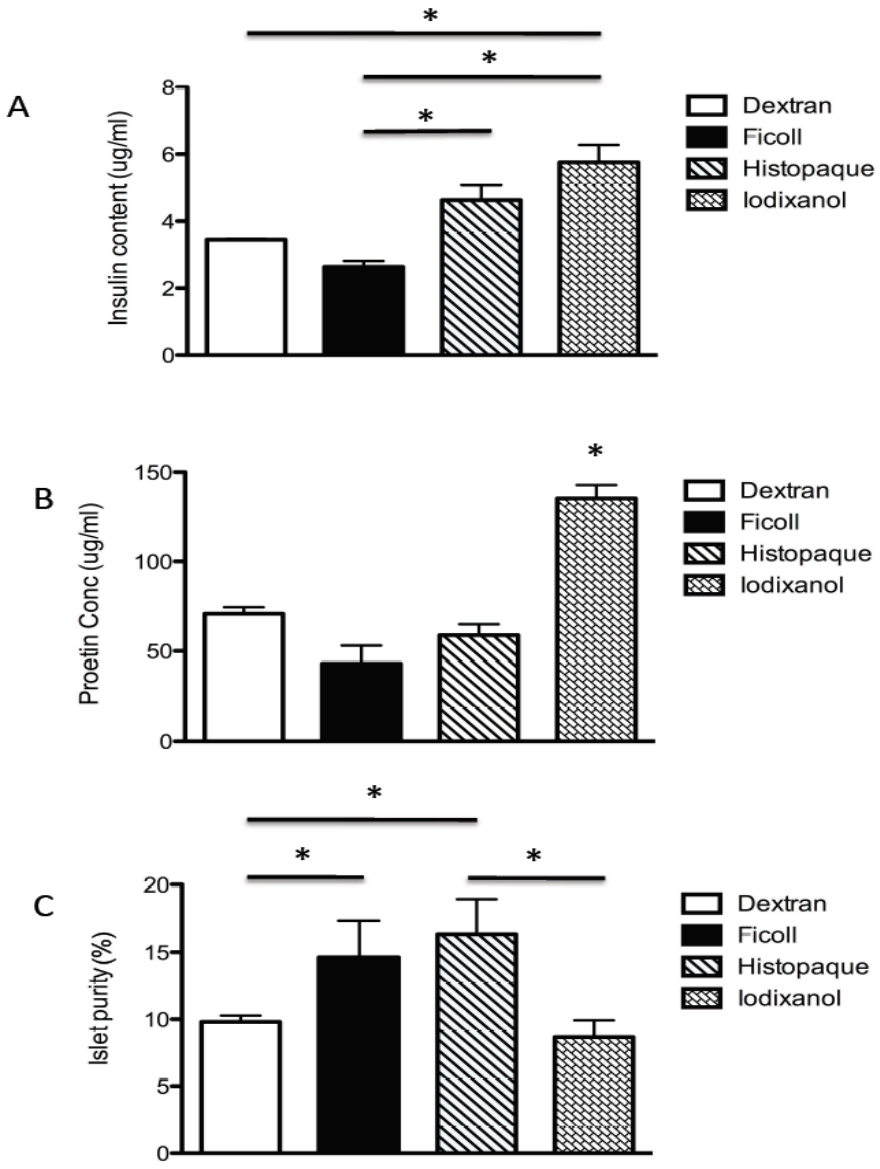
Sodium
Diatrizoate

Figure 2-2 The effect of islet isolation gradient composition on the number of islets isolated.



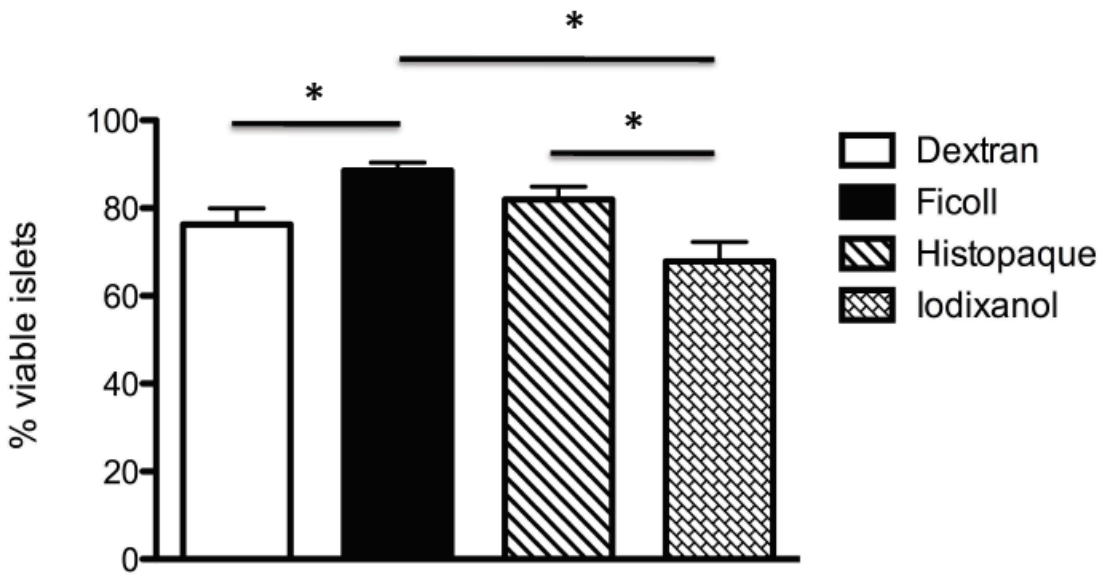
The number of islets per pancreas (A) was calculated by dividing the number of islets isolated on each gradient by the number of mouse pancreata these islets were harvested from. Islet equivalents (B) were calculated by normalizing the islets to a standard diameter of 150uM. The islet ratio (C) was calculated by dividing the islet equivalents by the total number of islets, giving an assessment of islet size. Counts were averaged over N=6 independent isolations. $p>0.05$ between groups.

Figure 2-3 Islet insulin content, protein content and purity after isolation on one of four gradients



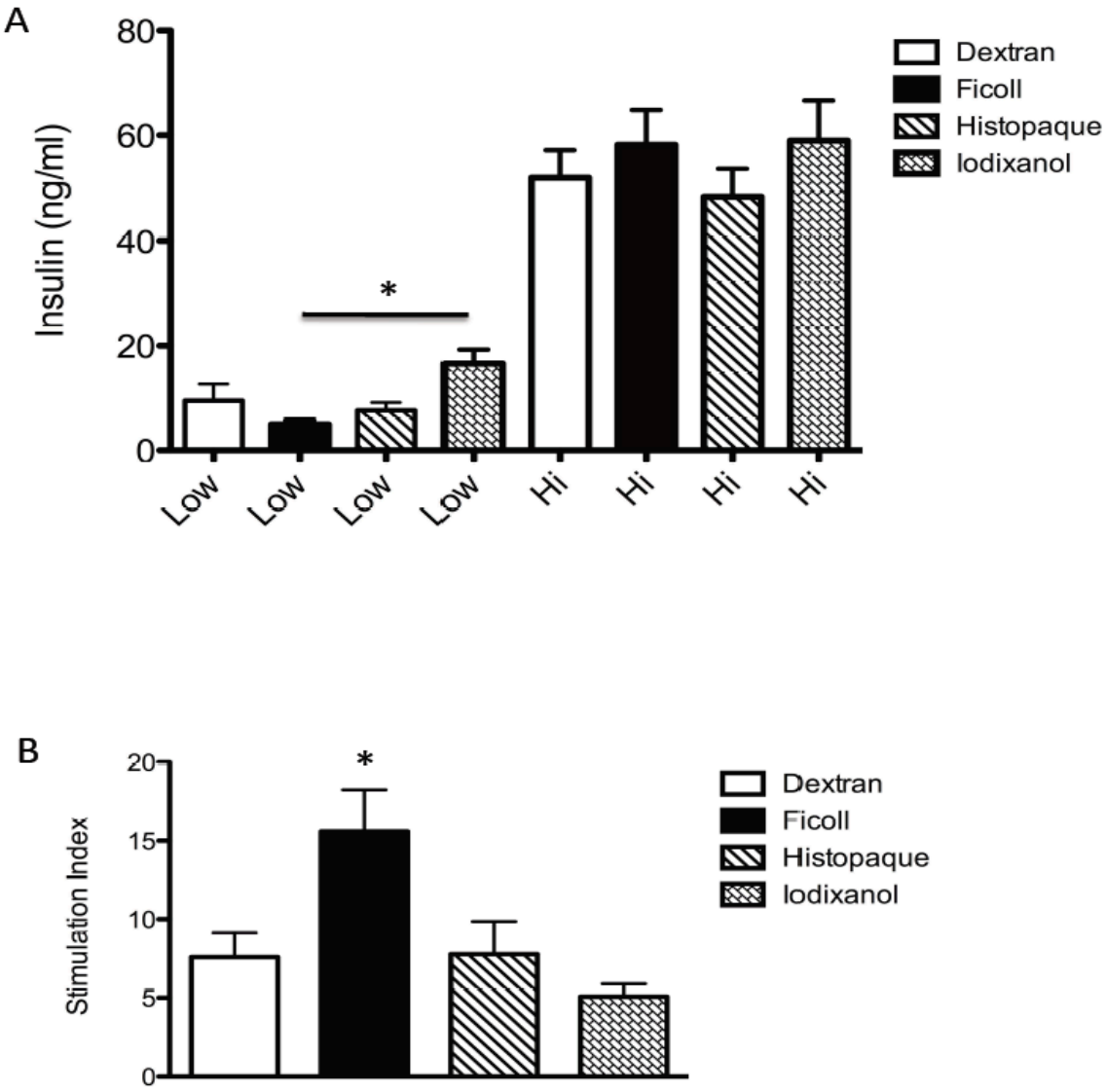
Two hundred and fifty (250) islets from each group were hand counted and frozen at -80°C until bulk analysis could be performed. Extraction was performed in acid-ethanol by homogenization and ultrasonic cell-membrane disruption. Insulin content of the neutralized extract was measured using a commercial ELISA kit (A). A further five hundred (500) islets were collected and stored at -80°C. The protein content of these islets was measured using a Bradford assay (B). The purity of the isolated islets was estimated by dividing the insulin content by the protein content (C). Analysis was performed in triplicate for each isolation over N=4 isolations. * $p<0.05$.

Figure 2-4 Islet viability immediately after gradient purification



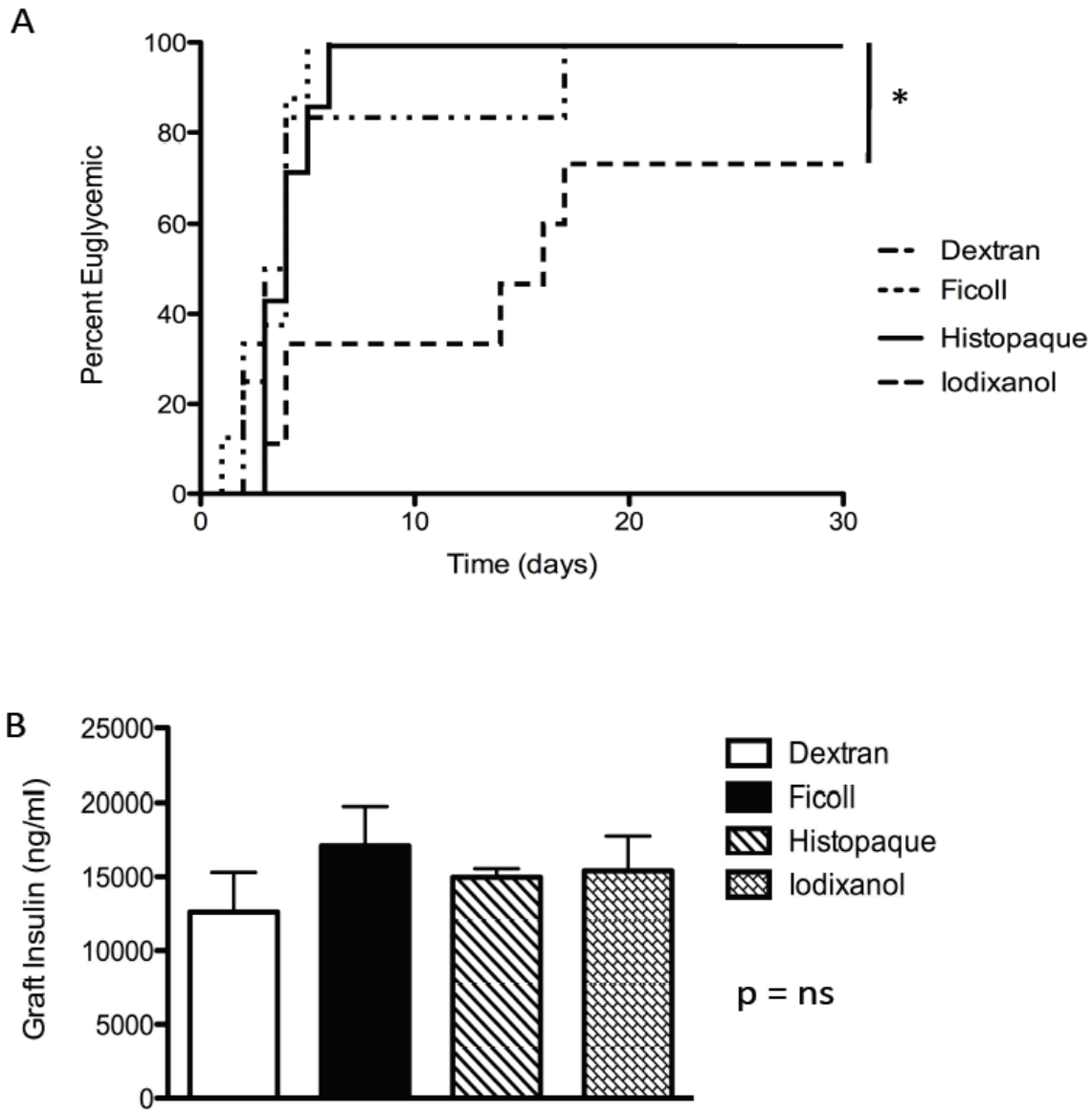
One hundred (100) hand-counted islets were subjected to viability assessment using Syto green/ethidium bromide staining under fluorescence microscopy (A). Triple aliquots were assessed by three independent observers and averaged for each isolation (N=6 isolations total). *p<0.05

Figure 2-5 Glucose-stimulated insulin release from purified islets



One hundred islets were collected and analyzed in duplicate from each gradient. Islets were washed in RPMI-1640 followed by 60min, 37°C incubations in low-glucose (RPMI-1640 with 0.56mg/ml D-glucose) and high-glucose (RPMI-1640 with 4.4mg/ml D-glucose) media. Supernatants were collected and analyzed on an ELISA kit (A). Stimulation index was calculated by dividing the mean insulin released from the high-glucose medium by the mean insulin released from the low-glucose medium (B). N=4 separate islet isolations were collected and analyzed separately and averaged. *$p<0.05$.

Figure 2-6 Diabetes reversal after islet transplantation



BALB/c mice were rendered diabetic with streptozotocin and transplanted under the kidney capsule with 500 spin-pelleted islets from one of the four gradients. (A) Kaplan-Meier analysis of diabetes reversal. Daily blood glucose measurements were taken via tail vein and mice were considered to be euglycemic with two consecutive readings <11mmol/L. Dextran: N=6, Ficoll: N=8, Histopaque: N=7, Iodixanol: N=9. (B) At the four week point, mice were euthanized and their graft-containing kidneys collected and the insulin extracted into acid-ethanol followed by neutralization. Insulin concentration of the supernatant was assessed using an ELISA kit. *$p<0.05$ by log-rank analysis, Ficoll versus Iodixanol.

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Chapter 3

Chapter 3a: Cell Death and Caspase Inhibition – a primer

Cell Death

Preventing early post-transplant death of islets is one of the mechanisms studied in this thesis as a means of enhancing islet engraftment and survival. While apoptosis, a form of programmed cell death, is the mode of cell death focused upon in the following studies, it is not the only form. The following sections serve to provide a brief introduction to cellular death and its inhibition through the use of caspase inhibitors.

Apoptosis

Apoptosis is a form of programmed cell death originally described by Kerr, Wyllie and Currie in 1972(*1*). They suggested that apoptosis is an endogenous, conserved program of cell death that works in concert with mitosis to control the size of a tissue or cell population. It can be initiated during normal tissue turnover, atrophy and involution but can also be triggered by insulting or noxious stimuli. The process of apoptosis is characterized by a reduction in cellular volume, chromatin condensation, DNA degradation (into segments which are multiples of 185 bp), preservation of organelle structure and plasma membrane integrity and cellular fragmentation into apoptotic bodies(*2*) (Table 3A-1).

Apoptosis is controlled by two pathways: the intrinsic pathway and the extrinsic pathway (Figure 3A-1). The extrinsic pathway is initiated by the binding of ligands (eg TNFα) to death receptors (eg TNF-R1). This leads to caspase-8 activation and subsequent effector caspase activation. By contrast, the intrinsic pathway is initiated by intracellular signals including DNA damage, hypoxia and endoplasmic reticulum stress which cause the release of pro-apoptotic signals from the mitichondria. Common to both of these pathways are the caspases, serine proteases with a specificity for aspartic acid residues which are activated and responsible for the characteristics of apoptosis(*3*). During apoptosis, the initiator caspases (caspases 2, 8, 9 and 10) are activated which in turn activate effector caspases (caspases 3, 6 and 7). These effector caspases initiate DNA fragmentation and organelle destruction. One key feature of an apoptotic cell is the transfer of phosphatidylserine to the plasma membrane. This marks the cell for removal by phagocytic cells, which do so in an orderly manner, avoiding an inflammatory response.

Necrosis

In contrast to apoptosis, necrosis is generally thought of as a pathological process in which cells in a living organism die with failure of membrane integrity (*4*) (Table 3A-1). The stimuli for necrosis are usually external to the cell and include infection, trauma and toxins. It is generally believed that necrosis incites a marked inflammatory response while not providing the correct signals to allow

phagocytes to respond and remove the cellular debris. The “point of no return” which renders necrosis irreversible is the loss of membrane integrity.

It should be clarified that necrosis refers to the correlate of cell death occurring in the presence of an irreversible exogenous injury. The most common manifestations are cell swelling, denaturation of proteins and breakdown of cellular organelles. This appearance of necrosis is due to two processes: enzymatic cell digestion and protein denaturation. Since these processes take hours to manifest, histologic evidence of necrosis may not be readily evident even though an irreversible injury has already occurred.

While the above refers to the “classical” view of necrosis as being an unregulated process, this definition has been increasingly questioned. Regulated necrosis, referred to as necroptosis, will be introduced below.

Caspase-independent cell death (CCID)

In addition to caspase-dependent programmed cell death, there exists a caspase-independent form(*5*). This is defined as death that ensues when a signal that normally induces apoptosis fails to activate caspases(*5*). While there are similarities to apoptosis including some of the upstream signaling pathways (including mitochondrial outer membrane permeabilization, MOMP), there are differences. These include the lack of phosphatidylserine externalization and wide-scale chromatin condensation; both caspase-dependent events.

Histologically, these cells exhibit cytoplasmic vacuolization, autophagosome accumulation and peripheral nuclear condensation. It has been shown that this method of cell death possesses much slower kinetics than apoptosis with a much wider variation in cellular characteristics(6).

The proof for CCID stems from both *in vitro* and *in vivo* models. Cell lines treated with the caspase inhibitor zVAD-FMK or those expressing genetically encoded caspase inhibitors are still able to induce apoptosis(7-10). Interdigital web loss is know to be a apoptosis-dependent process. The loss of *Apaf-1*, a key player in caspase-dependent apoptosis, slows but does not prevent interdigital web loss(11). In this model, CCID seems to compensate for a lack of apoptosis although it is unable to in other knockout models(12, 13). Although CCID occurs in these non-physiologic settings, there is also evidence that it has a physiologic role. Both cardiomyocytes and neurons display reduced levels of *Apaf-1* and shows signs of CCID after apoptotic stimuli(14-16).

Two mechanisms are hypothesized for how cells die in the absence of caspase activity(5). The first utilizes death receptors and is termed necroptosis. This was first observed when cells were shown to undergo cell death after death receptor ligation in the absence of caspase activation (17). This form of cell death can be facilitated by either phospholipase A_2 (by producing reactive oxygen species) or receptor-interacting protein-1 (RIP-1) (potentially through mitochondrial interaction) (18-20). The second mechanism of CCID involves mitochondrial

outer membrane permeabilization (MOMP). Direct induction of MOMP has been shown to induce cell death in the presence of caspase inhibitors(21). The main models by which MOMP may bring about cell death include a general decline in mitochondrial function or the release of mitochondrial proteins which can actively induce CCID(5).

Inhibition of apoptosis

Multiple forms of cell death exist although there is likely overlap between the pathways. In the studies to follow, islet apoptosis is focused upon as a dominant mediator of β-cell loss. In Chapter 3B and Chapter 4, a caspase inhibitor is utilized to prevent caspase-dependent apoptosis. The idea of using a caspase inhibitor to prevent apoptosis is certainly not a novel one. Caspase inhibitors are designed to mimic the binding site for the native caspases but contain an additional residue (eg. fluoromethylketone, FMK), which prevents its cleavage. Most caspase inhibitors are thus irreversible, however their selectivity for the various caspases differs.

The field of transplantation, where ischemia-reperfusion and apoptosis of donor cells/tissue play large roles in outcomes, has thoroughly explored caspase inhibitors in animal models over the last decade. One of the first studies utilized the caspase inhibitor acetyl-tyrosinyl-valyl-alanyl-aspartyl-chloro-methylketone

(Ac-YVAD-cmk) to increase survival of nigral transplants in rats (*22*). This was followed by numerous utilizing caspase inhibitors in lung(*23*), liver(*24, 25*) and kidney transplant models(*26*). The transition of these caspase inhibitors to the clinic has been slow. Safety has been a primary concern, particularly for the caspase inhibitor zVAD-FMK where the fluoromethylketone group could be converted to the toxic fluoroacetate in *vivo*(27*)*. That being said, these compounds still have enormous *in vitro* and pre-clinical *in vivo* potential. Even still, newer compounds including IDN-6556, the compound utilized in our studies, have proven safe in humans(*27-29*).

Inhibition of Islet Apoptosis

Apoptosis is the main mode of islet loss in the early post-transplant period, accounting for up to 60% of islet loss during the first few days following implantation (*30*). However, as outlined, caspase-dependent apoptosis is not the only form of cell death. Islets may undergo non-apoptotic forms of cell death which are not preventable by caspase inhibitors.

The idea of preventing β-cell apoptosis is not a new one. The overexpression of endogenous inhibitors of apoptosis including cFLIP (cellular FLICE-inhibitory protein; where FLICE=caspase 8) (*31-33*), A20 (an inhibitor of NF-κB which itself promotes apoptosis)(*32, 33*) and BCL-2 (*33-35*) have proven effective *in vitro* but not *in vivo*. These efforts focused either on the extrinsic or intrinsic caspase activation pathways and thus left the other pathway clear to initiate apoptosis and

required alteration of islets to achieve their results. Our lab has previously focused on inhibition of caspases pathway after the convergence of pathways; overexpressing X-linked inhibitor of apoptosis protein (XIAP) in islets leading to improved *in vitro* islet survival and post-transplant engraftment in a mouse model (*36, 37*). This would once again require alteration of islets to be effective in the clinic, however, these studies provided proof of principle that caspase inhibition can prevent β-cell apoptosis.

Our lab then turned to exogenous caspase inhibitors, drugs which could potentially be given to patients for a finite period after islet transplantation. Ideally one would choose a caspase inhibitor which inhibited caspases from both the extrinsic and intrinsic pathways or one which inhibited caspases after the pathways converged. Using either the caspase inhibitor zVAD-FMK or EP1013 Emamaullee et al. showed marked improvement in islet engraftment and a reduction in post-transplant β-cell apoptosis in mouse models(*38, 39*). These compounds were given to mice for 5-10 days post-transplant during the time of maximal islet apoptosis and loss. Unfortunately, while both of these compounds seemed promising for transition to the clinic, neither will make it that far due to safety concerns (z-VAD-FMK) unsecured rights and company closure (EP1013).

Figure 3A-1 The caspase activation pathway (including triggers and compounds to inhibit it) culminating in apoptosis

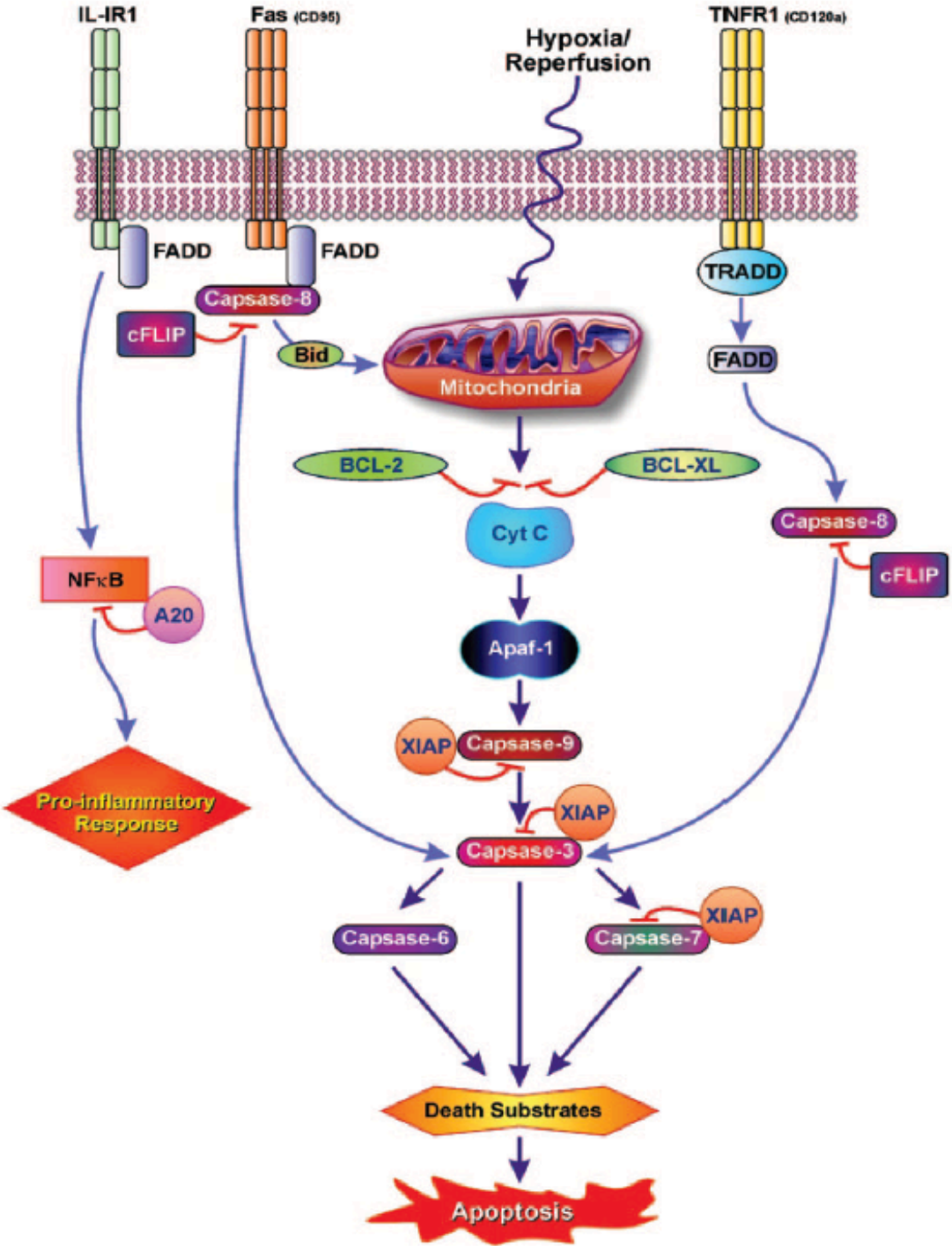

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Table 3A-1 Apoptosis versus Necrosis

	Apoptosis	Necrosis
Stimuli	Physiologic and pathologic factors	Hypoxia, toxins, infection; always pathologic
Histologic appearance	Single cells, chromatin condensation, apoptotic bodies	Sheets of cells, cellular swelling, organelle disruption
DNA fragmentation	Multiples of 185 bp	Random and diffuse
Mechanisms	Gene activation, endonucleases and proteases	Membrane injury, free radical damage
Membrane integrity	Maintained	Lost
Regulated?	Yes	No
Ingestion of dead cells	Neighboring phagocytes	Neutrophils and macrophages
Tissue reaction	No/minimal inflammation	Inflammation
Evolutionary conservation?	Yes	No

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Preface to Chapter 3b

Engraftment of islets after transplantation is a crucial step in their survival and functioning. It is known that up to **60% of islets are lost in the early post-transplant period**. This islet loss is mediated by **apoptosis** and necrosis. Our lab has previously explored caspase inhibitors as a means of preventing post-transplant apoptosis, a caspase-mediated event. Both zVAD-FMK and EP1013 (caspase inhibitors) proved effective in mouse models of diabetes and islet transplantation. However, neither of these compounds is transferable to the clinic (lost rights, company issues etc).

Idun Pharmaceuticals (then transferred to Pfizer Inc) has developed a compound named **IDN6556**, an **orally-dosed** caspase inhibitor with **high portal vein concentrations**. This compound has been explored in rodent models of liver inury and in clinical hepatitis where reduced levels of liver enzymes were observed.

The purpose of this study is to test IDN6556 in a mouse model of diabetes and islet transplantation. To do this, we employed a **marginal mass islet transplantation model** where a severely reduced number of islets was transplanted. This allowed us to study effects on islet engraftment by observing differences in diabetes reversal between treated and control mice. This model will be employed in subsequent chapters.

Chapter 3b: The Caspase Inhibitor IDN-6556 Improves Marginal Mass Engraftment after Islet Transplantation in Mice

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The caspase inhibitor IDN-6556 (PF3491390) improves marginal mass engraftment after islet transplantation in mice

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Background. *Islet transplantation has become a viable option for selected type 1 diabetic patients; however, a significant portion need to return to exogenous insulin. The predominant factors include impaired islet engraftment and early islet loss. Caspase inhibition is a potent way to improve islet engraftment, but all tested compounds so far have not been clinically relevant. IDN-6556 (PF3491390) has already been used clinically and can be delivered orally with high portal vein concentrations.*
Methods. *Mice were given a marginal mass islet graft of either mouse or human islets and treated with either IDN-6556 (10 or 20mg/kg ip bid) or vehicle and followed for diabetes reversal. At 1 month posttransplant, mice were subjected to a glucose tolerance test and an assessment of graft mass. In separate experiments, human islets were cultured with IDN-6556 or vehicle to assess for islet survival and viability.*
Results. *In both syngeneic mouse islets and human islets transplanted into immunodeficient mice, IDN-6556 (20mg/kg) given for 7 days posttransplant led to a significantly enhanced rate of diabetes reversal as compared to vehicle. In addition, mice receiving caspase inhibitor displayed improved glucose tolerance and graft survival at the 1-month point. We also found protective effects in vitro for islet viability and marked reduction in apoptosis in vivo.*
Conclusion. *Taken together, these results demonstrate the effectiveness of caspase inhibition with IDN-6556 on islet transplantation and in particular islet engraftment and survival. (Surgery 2011;■:■-■.)*

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ISLET TRANSPLANTATION has become a promising treatment for patients with labile type 1 diabetes. The introduction of the Edmonton Protocol in 2000[1] and the follow-up multicenter international trial[2] demonstrated that while insulin independence is achievable, there is a concern of deteriorating graft function over time.[3] In addition, while single-donor islet transplantation success has been achieved,[4,5] most centers still rely on multiple donor organs to achieve initial insulin independence. In fact, one of the main advantages of the Edmonton Protocol was the transplantation of an "adequate" islet mass, deemed to be >13,000 islet equivalents (IE)/kg of patient body weight.[1] This need for a large implant mass stems directly from the substantial islet loss observed within hours to days posttransplantation while the islets engraft. There are multiple insults an islet faces

Introduction

Islet transplantation has become a promising treatment for patients with labile type 1 diabetes. The introduction of the Edmonton Protocol in 2000(1) and the follow-up multi-center international trial(2) demonstrated that while insulin independence is achievable, there is a concern of deteriorating graft function over time(3). In addition, while single-donor islet transplantation success has been achieved(4, 5), most centers still rely on multiple donor organs to achieve initial insulin independence. In fact, one of the main advantages of the Edmonton Protocol was the transplantation of an “adequate” islet mass, deemed to be >13,000 islet equivalents (IE)/kg of patient body weight(1). This need for a large implant mass stems directly from the substantial islet loss observed within hours to days post-transplantation while the islets engraft. There are multiple insults an islet faces during this critical time including hypoxia, inflammation, instant blood mediated inflammatory reaction triggered by platelets after exposure to tissue factors and cytokines(6, 7). In fact, in both experimental and clinical islet transplantation, up to 70% of the implanted islet mass is lost in the first few days post-transplant(8, 9). In fact, a significant proportion of islets are lost within the first few minute to hours following implantation, as can be seen using PET-CT and radiolabeled islets(10). Maximizing islet survival in this critical stage would thus have enormous implications on clinical outcomes.

We have focused on inhibition of caspase pathways, to minimize apoptosis of islets and reduce early islet loss(11-14). IDN-6556 (PF3491390) a pan-caspase

inhibitor is a promising alternative (Pfizer, Cambridge, MA, USA). IDN-6556 is a highly selective pan-caspase inhibitor demonstrating irreversible inhibition and a significant first-pass effect(15). In fact, rodent studies showed roughly 6 times higher concentrations in the portal vein system than systemic after an oral dose, (15). A compound that is absorbed into the portal circulation at high concentration would be particularly attractive for islet transplantation, where clinically, islets are implanted intraportally and immunosuppressants reach levels 2 – 10 times those seen systemically(16, 17). In the present study, we sought to determine the effectiveness of IDN-6556 in both syngeneic mouse islet transplantation and human islet transplantation in immunodeficient mice.

<u>Hypothesis</u>: The caspase inhibitor IDN6556 will enhance engraftment of islets in a mouse model of diabetes through prevention of early post-transplant apoptosis.

Research Design and Methods

Animals and Reagents.

Immunodeficient B6-RAG $^{-/-}$ mice (B6.129S7-*Rag1tm1Mom*/J) were obtained from the Jackson Laboratories (Bar Harbor, ME) and housed under specific pathogen-free conditions. BALB/c mice were also obtained from the Jackson Laboratories but housed under conventional conditions. All animals were cared for according to the guidelines of the Canadian Council on Animal Care, and ethical approval was obtained from the animal welfare committee at the University of Alberta.

All reagents were obtained from Sigma Aldrich (Oakville, Ontario, Canada) unless otherwise specified. IDN-6556 (PF3491390) was provided as a generous gift from Pfizer (Cambridge, MA, USA).

Mouse Islet Isolation

Mouse islets were isolated using established protocols with minor modifications(18). In brief, mouse pancreata were digested with collagenase (1.0mg/ml in Hanks' buffered saline solution (HBSS)) and purified with Histopaque-density centrifugation. Handpicked islets were washed with HBSS then placed in short-term culture in Connaught Medical Research Laboratories (CMRL-1066) medium supplemented with 10% fetal bovine serum, L-glutamine (100 mg/l), penicillin (112 kU/l), streptomycin (112 mg/l) and HEPES (25 mmol/l). Islets were cultured for a maximum of two hours before transplantation.

Human Islet Isolation

Pancreata were retrieved from multiorgan deceased donors after aortic cross-clamp and infusion of Histidine-Tryptophan-Ketoglutarate (HTK) solution. Islets were isolated according to a modified Ricordi's semi-automated technique(19, 20). Briefly, the pancreas was distended with collagenase NB1 supplemented with neutral protease (Serva Electrophoresis GMbH) and digested in a Ricordi chamber. When free islets were released, tissue digest was collected and further purified on a cell sorter (Model 2991, Cobe, Lakewood, CO) using a continuous density gradient(21). Human islets were cultured in CMRL-1066 (as above) for 72 hours at 37°C before transplantation into diabetic mouse recipients.

Islet Transplantation

Streptozotocin was administered to recipient mice to induce diabetes (BALB/c: 220mg/kg i.p; B6-RAG$^{-/-}$: 180mg/kg i.p). Animals were considered diabetic after two consecutive blood glucose measurements ≥ 20 mmol/L using a OneTouch Ultra glucometer (Lifescan Canada, Burnaby, B.C). For mouse islet studies, a marginal mass of 150 islets were implanted into the kidney subcapsular space. In human islet studies, an equivalent marginal mass of 1500 islet equivalents (IE) were implanted beneath the kidney capsule. Transplant recipients were given a twice-daily intraperitoneal injection of either vehicle (PBS, 100uL) or IDN-6556

(10mg/kg or 20mg/kg) on the day of transplant and daily for the first seven days post-transplant (to coincide with the period of maximal apoptosis).

Glucose tolerance tests

Transplanted mice were fasted for 16-20 hours and injected intraperitoneally with 50% dextrose at 2g/kg body weight (intraperitoneal glucose tolerance test, IPGTT). Blood glucose levels were analyzed at baseline, 5, 15, 30, 60, 90 and 120 minutes post-injection.

Graft insulin content

Islet grafts were harvested from the kidney capsule and stored at -80°C until bulk analysis could be performed. Extraction was performed in acid-ethanol by homogenization and ultrasonic cell membrane disruption. Insulin concentration of the neutralized extract was measured using a commercial ELISA kit (Alpco Diagnostics, Windham, NH).

Human Islet Culture and viability

A portion of each human islet isolation was placed in culture in CMRL-1066 at 37°C. To half of the islets was added 100uM PF-3491390 and to the other half an equivalent volume of PBS (control). Islets were counted using dithizone at the beginning of culture and at the 48 hour point. Viability was assessed using Syto

green/ethidium bromide, couting 100 islets under fluorescence light microscopy as previously described (Cedarlane laboratories and Sigma-Aldrich, ON, Canada)(22, 23).

Apoptosis Assays

Apoptosis of islet cells within transplanted grafts was quantified using TUNEL staining (Dead-end Apoptosis Detection System, Promega, Madison, WI) Nuclear counterstaining with DAPI (Molecular Probes,
Eugene, OR) was used to detect all cells present in the sample. Islet grafts were harvested, placed in formalin, processed and embedded in paraffin. To quantify apoptosis in vivo, fields containing at least 500 cells were analyzed at 200x magnification. The number of TUNEL+ cells (green) within the insulin+ islet graft area of the section were counted and compared to the total number of DAPI+nuclei within that same field to determine % apoptosis. Sections were prepared from 3 transplant recipients in each cohort, and at least 3 fields were analyzed in each section.

Statistics

Data was analyzed using GraphPad Prism (Version 5.0b, GraphPad Software Inc., San Diego, CA). P values less than 0.05 were considered statistically significant. Graphical representation of data is represented as mean ± SEM, unless otherwise indicated in the figure legends. Column means were compared

using the Mann-Whitney test while survival analysis was carried out using log-rank analysis.

Results

IDN-6556 improves diabetes reversal in a marginal mass syngeneic islet model

Diabetic BALB/c mice, transplanted with a marginal mass of syngeneic islets under the kidney capsule, were treated with IDN-6556 at either 10mg/kg or 20mg/kg twice daily intraperitoneally for one week (Figure 3-1). Only mice treated with 20mg/kg showed a significantly higher rate of diabetes reversal as compared to control mice (Figure 3-1B) (61.2% versus 28.8%, $p<0.05$ by log-rank analysis). As such, 20 mg/kg was used as the dose for further studies.

In a syngeneic mouse islet transplant model, islets display improved in vivo function and survival when the host is treated with IDN-6556

To determine the effect of IDN-6556 on islet function, transplanted mice displaying non-fasting blood glucose readings <18mmol/L were subjected to an IPGTT four weeks after islet transplantation. Mice receiving IDN-6556 (20 mg/kg) for one week post-transplant displayed improved glucose tolerance (Figure 3-2A) which was reflected in a significantly smaller area under the curve (Figure 3-2B, $p<0.05$ versus control)

After allowing 48 hours for graft recovery after the IPGTT, mice were sacrificed and their graft-bearing kidneys were recovered in order to determine the surviving islet mass. Mice receiving IDN-6556 has significantly increased graft

insulin content at the study endpoint as compared to control mice (Figure 3-3, $p<0.05$). After the graft bearing kidney was removed, mice were monitored for 24 hours to confirm return to diabetes. In all cases, mice showed blood glucose readings >18mmol/L within 24 hours (data not shown).

Human Islets cultured with IDN-6556 display a higher yield and improved viability in vitro

A portion of each human islet isolation was kept aside for *in vitro* analysis. Islets were counted initially then placed in culture with either IDN-6556 at 100uM or vehicle (PBS) as a supplement. At 48 hours, islets were re-counted and assessed for viability. Four separate and independent islet preparations were received. In each case, the percentage of islets remaining after 48 hours was higher in the portion co-cultured with IDN-6556 (48-73% versus 21-62% for control) (Figure 3-4A). In addition, islet viability was enhanced at 48 hours as assessed by Syto green/ethidium bromide analysis (83% versus 72%, $p < 0.05$) (Figure 3-4B).

IDN-6556 treatment leads to superior diabetes reversal when given to immunodeficient mice receiving a marginal mass of human islets

To confirm that the positive effect on mouse islets was applicable to human islets, diabetic B6-Rag$^{-/-}$ mice were transplanted with a marginal mass of human islets and treated with either IDN-6556 (20mg/kg) (n=17) or control (n=10). Mice receiving the caspase inhibitor for the week following islet transplantation showed

a significantly higher rate of diabetes reversal as compared to control mice (Figure 3-5, 87.5% versus 30%, $p<0.01$ by log-rank analysis).

IDN-6556 leads to enhanced function and survival of human islets in vivo

Immunodeficient mice with non-fasting blood glucose measurements <18 mmol/L were subjected to an IPGTT (Figure 3-6). Mice treated with IDN-6556 (N=12) in the post-transplant period displayed reduced post-dextrose blood glucose levels (Figure 3-6A) and an improved area under the curve (Figure 3-6B, $p<0.05$) as compared to controls (N=7).

At the study endpoint (one month) mice were sacrificed and their grafts recovered for insulin content. After homogenization and sonication, supernatants were analyzed for their human insulin content using a commercially available ELISA kit. Once again, the mice treated with the caspase inhibitor displayed a 2.6-fold enhancement of insulin content in their grafts compared to control (Figure 3-7, $p<0.05$).

IDN-6556 acts through inhibition of apoptosis

BALB/c mice were rendered diabetic using streptozotocin and transplanted with 500 syngeneic islets under the kidney capsule. Mice (n=3 per group) were treated with either IDN-6556 (20mg/kg i.p bid) or control for 24 hours at which

point the mice were euthanized and their grafts recovered, fixed and TUNEL assays completed as described above. Grafts from mice treated with IDN-6556 showed significantly less apoptosis 24 hours after transplantation (4.02%) than control mice (51.12%, $p<0.05$) (Figure 3-8). This is in keeping with prior results from our laboratory with alternative caspase inhibitors(13).

Discussion

The present study has demonstrated the potency of the caspase inhibitor IDN-6556 to facilitate engraftment of both mouse and human islets, leading to improved islet function and survival in vivo. In addition, we have shown positive effects on islets in culture where survival and viability were enhanced.

Islet engraftment is a crucial part of the islet transplantation process. It is already known that large islet losses can occur during this time due to a number of interrelated reasons including hypoxia and inflammation. It is likely that these insults trigger the common pathway of islet apoptosis. An opportunity to intervene and prevent apoptosis could potentially lead to enhanced islet engraftment and survival. To achieve maximal benefit, we hypothesize that apoptosis blockade is required only temporarily during the initial period of islet engraftment. By limiting this exposure to acute dosing over a period of 7 days, potential concerns of long-term caspase inhibition in heightening risk of malignancy and other systemic toxicities can be substantially minimized or eliminated. In this study, caspase inhibitor therapy was tailored to provide maximal impact during the engraftment period but to minimize the risk of side-effects. No systemic side-effects were observed, and no malignancies developed during the limited period of follow-up.

Two synthetic peptidyl caspase inhibitors, *N*-benzyloxycabonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD-FMK) and EP1013 (zVD-FMK) have been explored previously by our laboratory as means of improving islet survival during engraftment(13, 14). While results with these compounds have been promising, showing benefits to islet engraftment in mouse models, neither are transferable

to the clinic secondary to unsecured property rights, safety concerns with fluoromethylketone radicals (zVAD-FMK) and company closure (EP1013). IDN-6556, a highly selective caspase inhibitor has shown promising effects on islet engraftment in this study and is particularly appropriate in clinical islet transplantation due to its oral bioavailability. Clinically, IDN-6556 was shown to lower aminotransferase levels in hepatitis C patients(24) and to provide protection against cold and warm-ischemia induced hepatic injury in liver transplantation(25).

While these positive results on mouse islets are encouraging, showing benefits to human islets is an important requisite for potential clinical translation. The use of a marginal mass islet model is highly representative of the clinical setting, where a patient receiving a single donor islet mass is less likely to become insulin independent without optimal islet preparation and intensive post-transplant therapy(4, 5). In a setting free of immune-mediated islet attack we have shown that the addition of the caspase inhibitor IDN-6556 leads to a 57% higher diabetes reversal rate. In addition, this reversal is robust with treated mice displaying improved islet functionality and islet survival.

Of substantial relevance is the additional finding that IDN-6556 co-culture with human islets leads to improved islet survival. One of the essential components of the original Edmonton Protocol success was the implantation of a large and sufficient mass to achieve insulin independence(1). However, not all islet

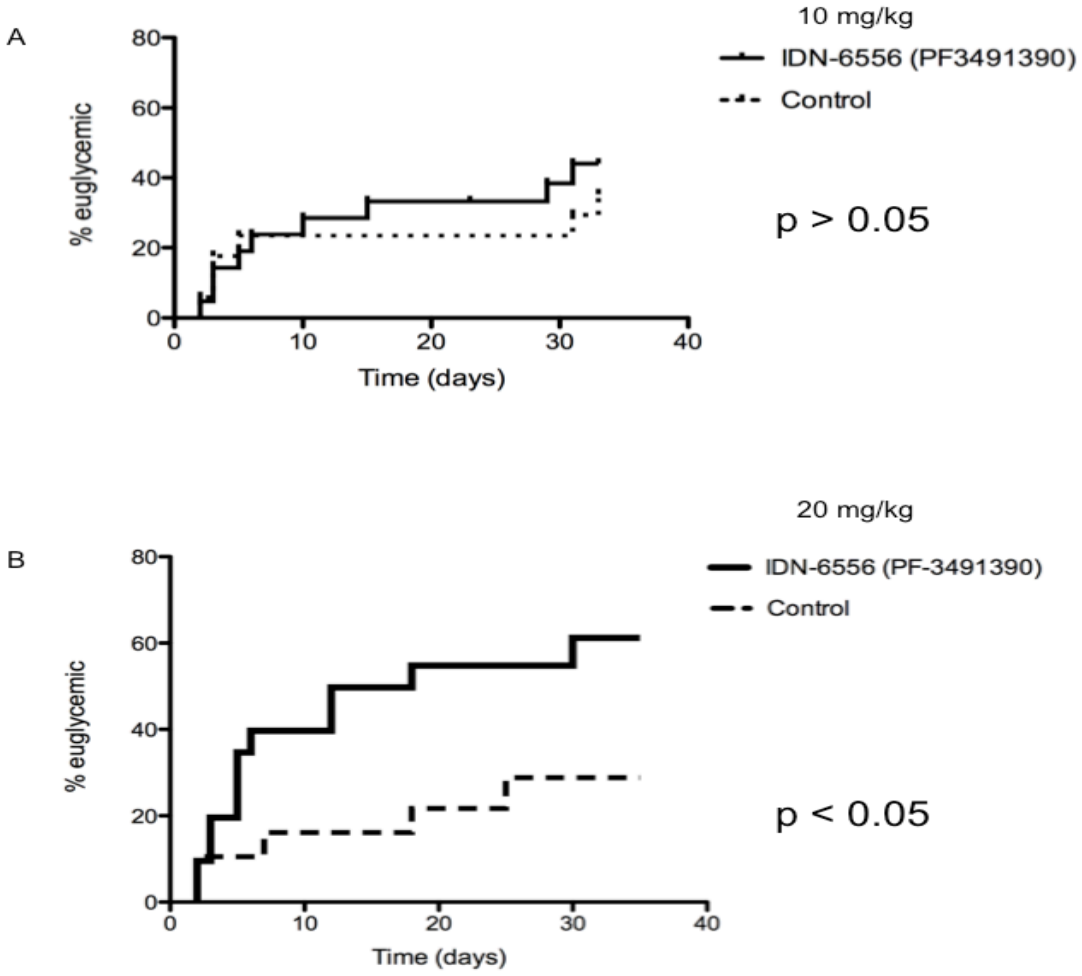
preparations are adequate for clinical transplantation. A recent study showed that only 23.5% of 241 human islet preparations contained >300,000IE, in other words a large proportion of islet isolations failed to generate sufficient islet minimal transplantable mass threshold of 5,000IE/kg(26). The majority of islet preparations are cultured, once purified, in order to facilitate islet shipping and patient preparation. Even twenty hours of culture can negatively affect islet yields with at least 35% of islet preparations experiencing a fall in islet mass, and with a mean islet mass loss of approximately 20% seen overall(19). Co-culturing of clinical islets with IDN-6556 could prevent this islet loss and potentially allow an increased number of preparations to meet the cut-off for clinical transplantation. Furthermore, by avoiding exposure of the recipient to damaged and inflamed islets, the potential risk of broad HLA-sensitization could be lessened, and the opportunity to facilitate immunological tolerance induction could be enhanced(27-29).

The current compound, IDN-6556, is herein demonstrated to profoundly protect both murine and human islets in culture and after transplantation, and offers the additional potential of oral administration, and has been shown to have heightened concentrations in the portal vein system(15). In the present study, we did not utilize the portal vein but rather the renal subcapsular space for islet implantation, as we find the latter model to be less variable and more consistent in the context of a marginal mass murine model. The portal implantation site in mice typically requires a higher number of islets to achieve diabetes reversal, is

more technically difficult, and has a higher post-operative mortality rate(30). Recovery of islet grafts at the study endpoint for insulin quantification also becomes challenging than the more localized renal subcapsular graft, although not impossible(31). The ability of this caspase inhibitor to display engraftment benefit at this non-portal site shows its enhanced potency; we could expect even more drastic effects with islets transplanted into the liver and oral drug dosing. In fact, further studies are currently underway in our laboratory to investigate this caspase inhibitor in a large animal pig islet autotransplant model using oral drug dosing and portal vein islet implantation. Finally, the IDN-6556 compound has already been tested clinically in the setting of liver transplantation and in the presence of full systemic immunosuppression, and was well tolerated without risk of malignancy or systemic toxicity in a limited early treatment study where this compound reduced risk of ischemia-reperfusion injury(25).

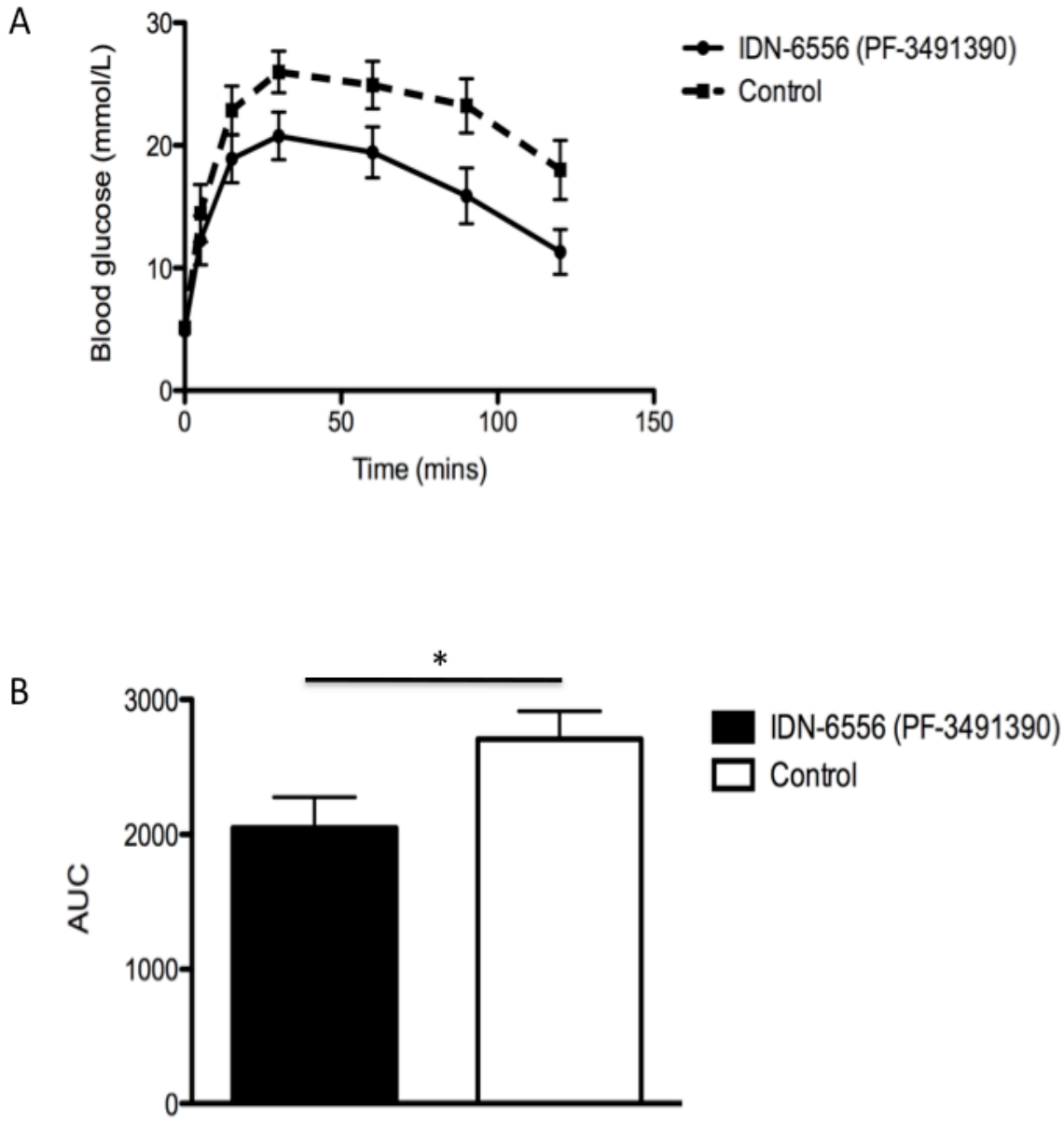
Overall, this study demonstrates that the caspase inhibitor IDN-6556 is able to facilitate both mouse and human islet engraftment in mice through the inhibition of apoptosis, leading to substantial improvement in islet survival and function. It also promotes human islet survival in culture. These results require further validation in a clinically relevant large animal model of diabetes before this compound is brought forth to the clinic.

Figure 3-1 IDN-6556 (PF3491390) promotes islet engraftment and diabetes reversal in marginal mass syngeneic islet transplantation



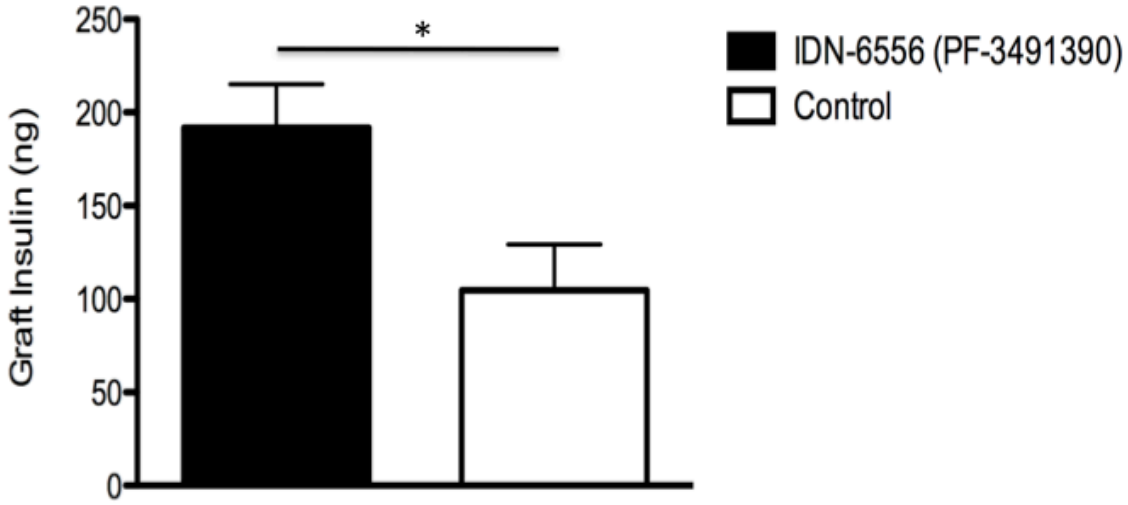
Marginal mass islet grafts consisting of 150 syngeneic islet were transplanted under the kidney capsule of streptozotocin-induced diabetic BALB/c mice. In order to determine a therapeutic dose of IDN-6556 mice were given either IDN-6556 10 mg/kg ip twice daily (bid) or 20 mg/kg ip bid for seven days and compared to control (phosphate buffered saline) in each case. Blood glucose readings were taken daily for the first week and then three times weekly until the endpoint of four weeks. A return to euglycemia was defined as two consecutive blood glucose readings ≤ 11mmol/L. (A) IDN-6556 10mg/kg ip bid for seven days (N=21) did not lead to an improvement in diabetes reversal when compared to control (N=17) ($p>0.05$) (B) In comparison, IDN-6556 at 20 mg/kg led to euglycemia at a significantly increased rate (61.2%, n=21 versus 28.8% n=19, $p < 0.05$ by log-rank analysis).

Figure 3-2 IPGTT. IDN-6556 leads to improved glucose tolerance one month after syngeneic marginal mass islet transplantation



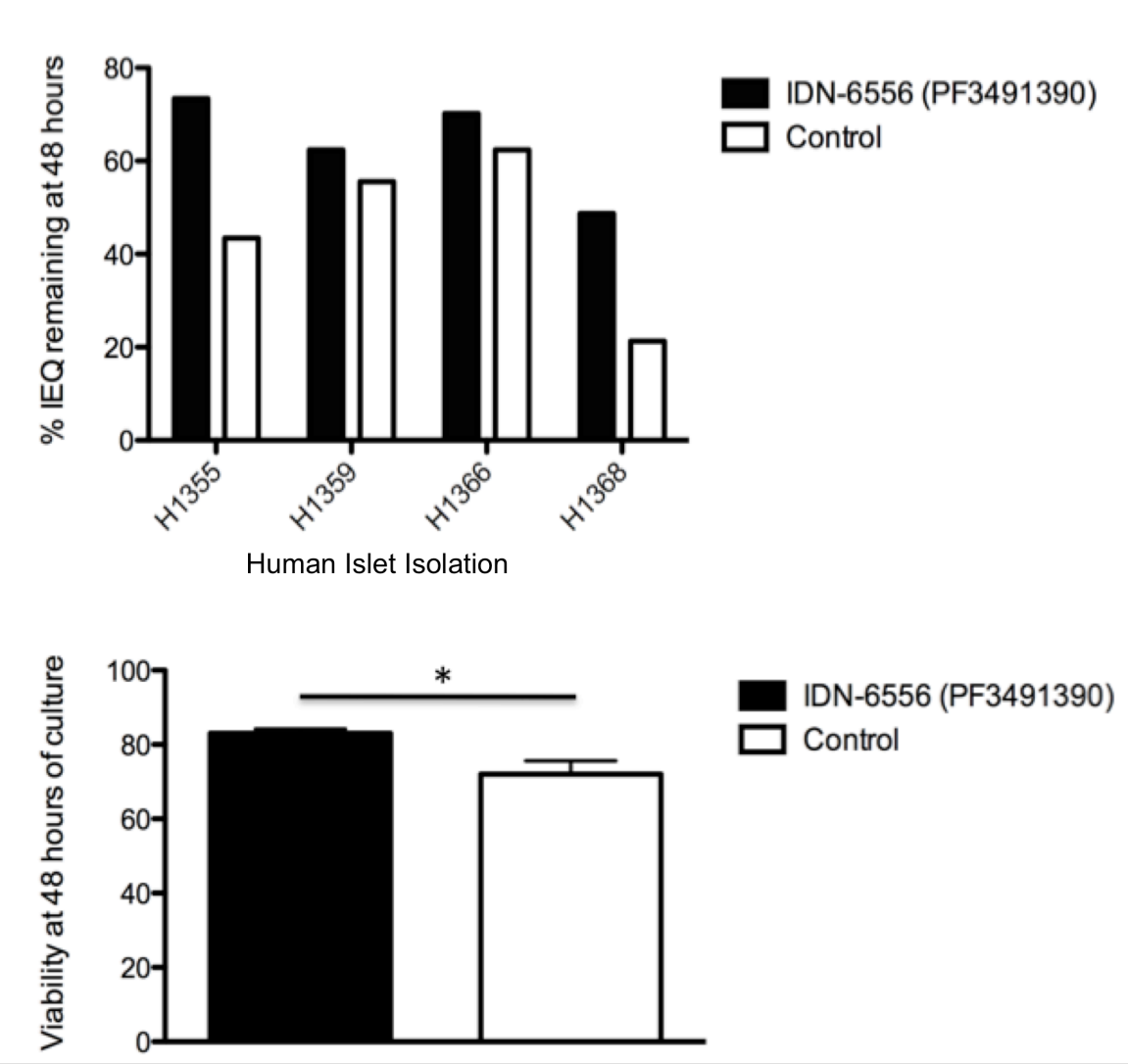
BALB/c mice receiving marginal mass syngeneic islet grafts (150 islets) one month previously and displaying non-fasting blood glucose readings < 18mmol/L (IDN-6556: N=14, Control: N=12) were fasted for 18 hours and given 3g/kg of dextrose ip followed by blood glucose sampling. (*A*) IDN-6556 treatment for one week after islet transplantation led to reduced blood glucose levels after a dextrose bolus which translated into a significant reduction in the area under the curve (AUC) (B) (* = $p<0.05$).

Figure 3-3 Mice treated with IDN-6556 display improved islet graft survival



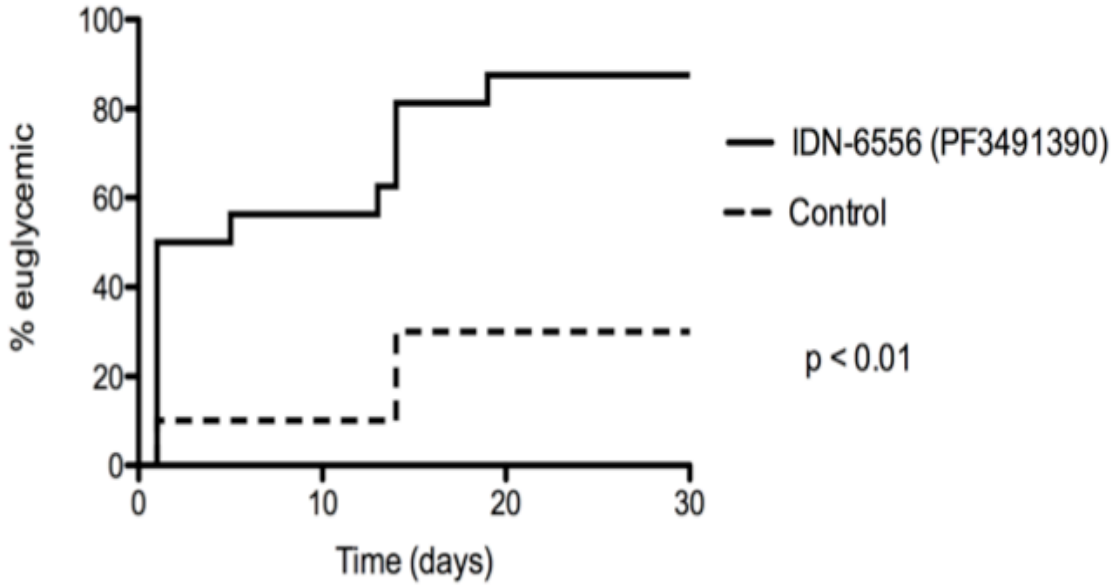
At the four-week point, mice were sacrificed and their graft-bearing kidneys were recovered. After cold storage, grafts were homogenized and sonicated before the supernatant was analyzed using an ELISA kit. IDN-6556 treatment led to a significant improvement in insulin content of the graft as compared to mice receiving vehicle (* = $p<0.05$).

Figure 3-4 Human Islets cultured with 100mM IDN-6556 display improved *in vitro* survival and viability



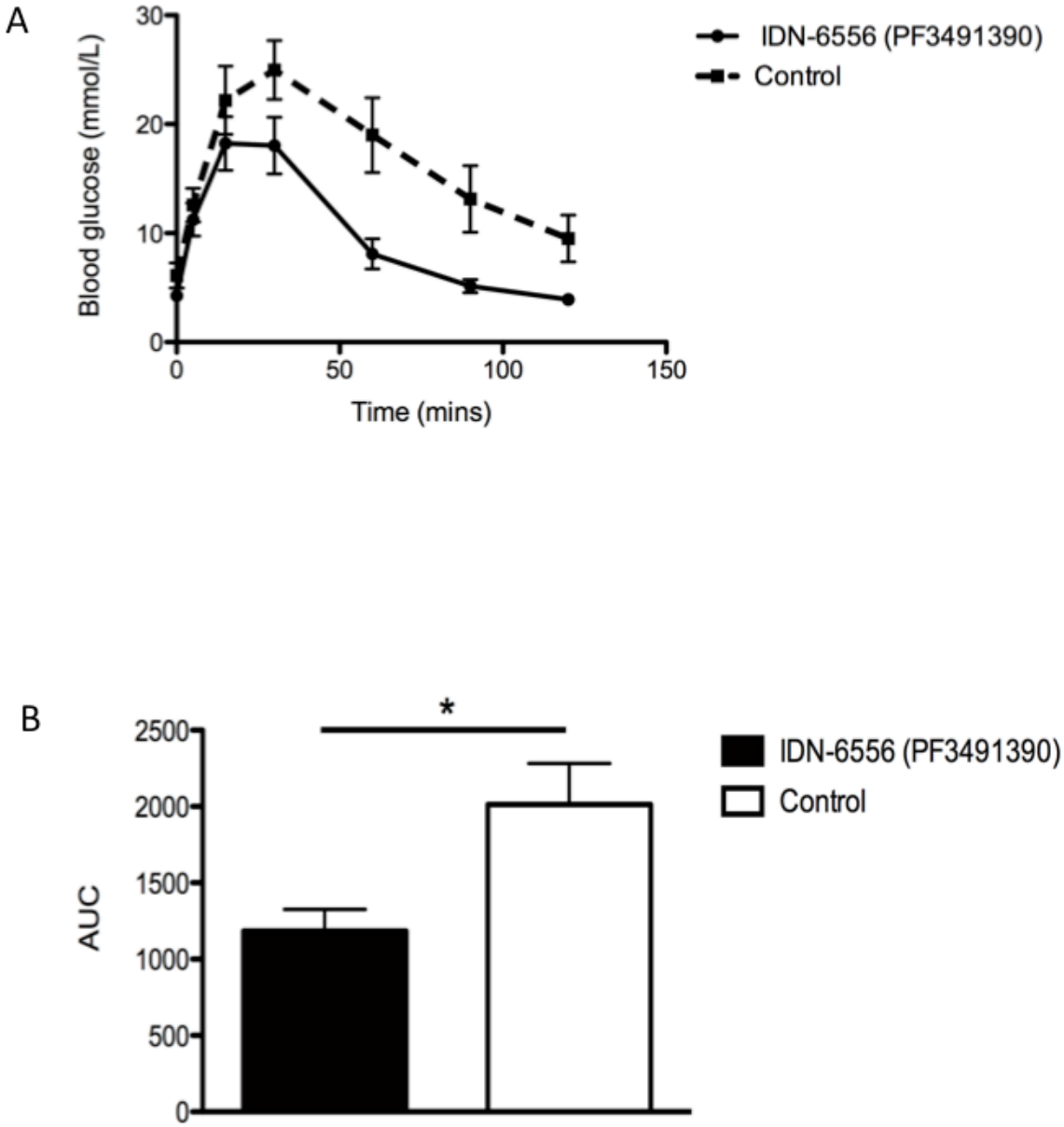
Human islets were cultured in CMRL 1066 media with either 100mM IDN-6556 or vehicle (PBS). After 48 hours of culture at 37°C and 5%CO_2, islets were counted using dithizone staining and the viability of 100 islets assessed using syto green/ethidium bromide. Co-culture with IDN-6556 led to survival of a higher number of islet equivalents in all islet preps (H# refers to human islet preparation) (A) with improved viability at 48 hours (B) (* = $p<0.05$).

Figure 3-5 IDN-6556 leads to improved diabetes reversal in a marginal mass human islet model



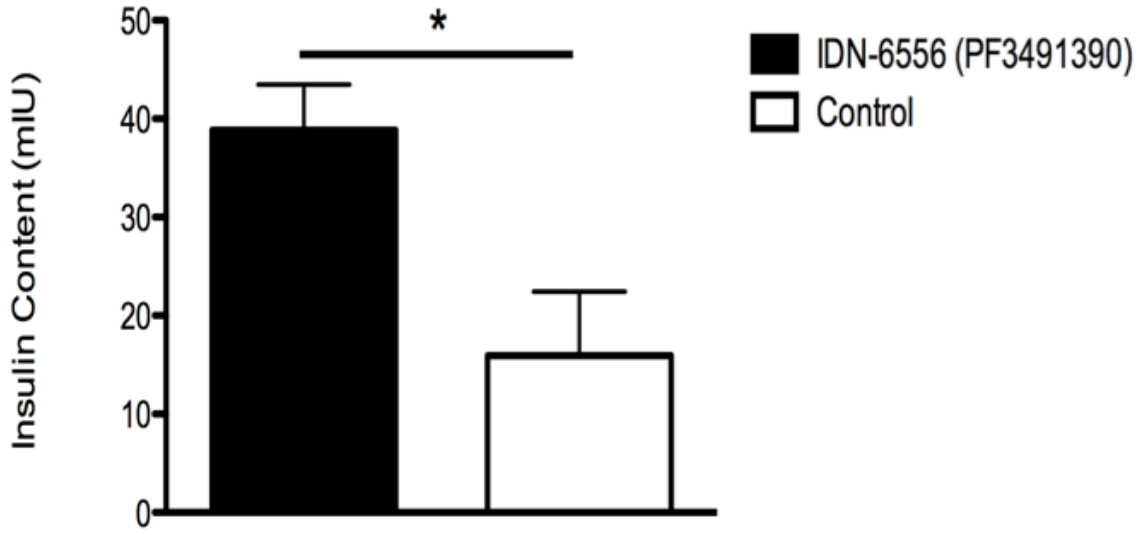
B6-Rag $^{-/-}$ mice were rendered diabetic using streptozotocin then transplanted with a marginal mass of human islets (1500 islet equivalents) under the kidney capsule. Mice were split into two cohorts and treated with either IDN-6556 (20mg/kg ip bid, N=17) or as controls (PBS, 100ul ip bid, N=10) on the transplantation day and for seven days thereafter. IDN-6556 treatment led to a significantly higher rate of diabetes reversal as compared to control (87.5% versus 30%, $p<0.01$).

Figure 3-6 Intraperitoneal Glucose Tolerance Test



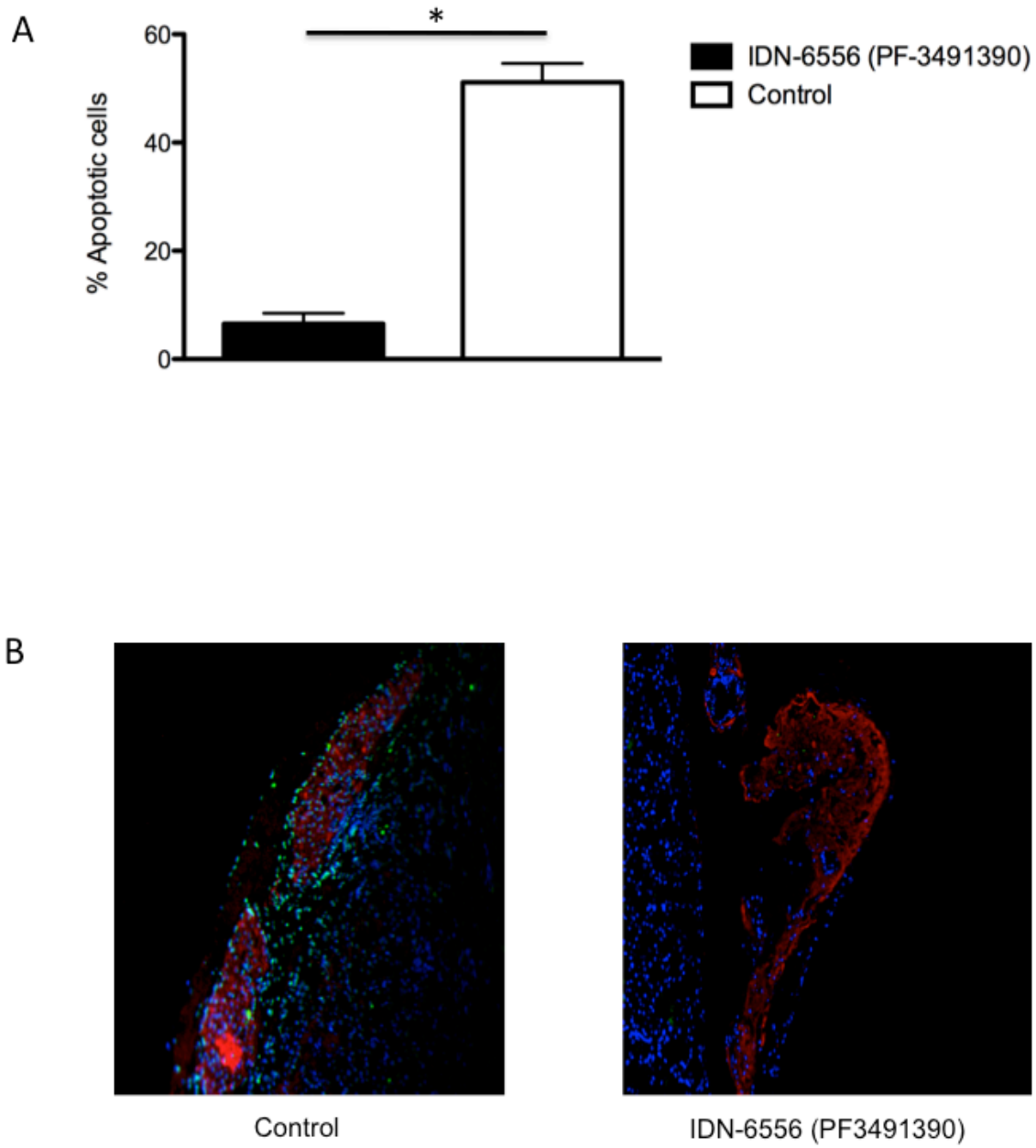
One month after islet transplantation, mice receiving a marginal human islet mass graft with non-fasting blood glucose readings < 18mmol/L were fasted for 18 hours and given a 3g/kg ip bolus of dextrose. (*A*) Mice receiving IDN-6556 (N=12) in the post-transplant period showed lower blood glucose readings versus control (N=7) and (*B*) a significantly reduced AUC (* = $p<0.05$)

Figure 3-7 IDN-6556 leads to improved human islet survival in diabetic immunodeficient mice



At the study endpoint (4 weeks) mice were sacrificed and their graft bearing kidneys recovered. These were acid-ethanol homogenized and sonicated to extract the insulin which was quantified using an ELISA kit. Mice that received IDN-6556 is the post-transplant period displayed an increased graft human insulin content (* = $p<0.05$)

Figure 3-8 IDN-6556 treatment reduces apoptosis in islet grafts 24hours after transplantation



Twenty four hours after transplantation of 500 syngeneic islets under the kidney capsule, BALB/c mice treated with either IDN-6556 or vehicle (N=3 per group) were euthanized and their grafts harvested. These were fixed in formalin, embedded in paraffin and underwent a TUNEL assay. The number of TUNEL+ (green) cells were counted and are displayed as a percentage of all cells counted (DAPI+) within the Insulin+ graft (A). At least N=3 sections were analyzed per graft, counting 500 DAPI+ cells in each. Representative sections are displayed in (B) (TUNEL = green, Insulin = red, DAPI = blue)

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Chapter 4

Preface to Chapter 4

The results of the previous chapter show **IDN6556 to be an effective means of preventing early islet apoptosis and enhancing islet engraftment**. This certainly has the **potential to benefit clinical islet transplantation**. However, we did not employ the portal vein site nor did we administer the caspase inhibitor orally in our mouse study.

The purpose of this chapter is to study the effects of IDN6556 in a large animal model as a bridge to clinical trials. **Total pancreatectomy** will be used to confer diabetes followed by **marginal mass islet autotransplantation**. We will **ensure complete pancreatectomy** by measuring arginine-induced insulin released immediately post-pancreatectomy. This study was **not designed as a long-term study**. We wanted to observe the initial effects (up to one month) of a one week course of orally-dosed IDN6556. **Our main endpoints include achievement of euglycemia and glucose tolerance at one month**.

The Caspase Inhibitor IDN6556 Facilitates Marginal Mass Islet Engraftment in a Porcine Islet Autotransplant Model

A version of this chapter has been submitted to *Transplantation* for publication

Introduction

Islet transplantation has become a promising treatment option for select patients with type 1 diabetes. Since 2000 there have been an estimated 700 islet allotransplants performed worldwide, with combined results of three leading centers reporting insulin independence rates of 82% at one year (1-3). Most centers, but not all, report the need for more than one donor to achieve sufficient islet engraftment mass, and rates of insulin independence have waned over time, with 55% remaining insulin free at two years, and as low as 15% by five years (4). The current paradigm of success is sufficient human c-peptide secretion to to protect from hypoglycemia and correct hemoglobin A1C, even if this falls short of complete insulin independence. A major goal is therefore to augment the initial islet engraftment mass, and provide sustained immunological protection from auto- and allorejection, and several promising strategies have emerged to promote this. It is known that up to 70% of the transplanted islet mass is lost within the initial hours to days post-transplant (5-9). The instant blood-mediated inflammatory reaction, hypoxia, delayed revascularization, reperfusion, and inflammatory cytokines all contribute to early islet loss culminating in islet apoptosis or necrosis. This process is initiated in the donor, exacerbated during cold ischemic storage and during islet isolation and subsequent culture, and persists during the early engraftment phase in the recipient portal venous system (5, 10-13).

In the present study, we investigate the potential protective effect of a potent pan-caspase inhibitor, IDN6556 (formerly Pfizer Inc, and recently Conatus Pharmaceuticals Inc, San Diego, CA), as a means to prevent apoptosis of intraportal islet transplants in a porcine autograft large animal model. We previously reported potency of this compound in protecting marginal mass syngenic intraportal islet transplants in mice, and similarly with human islets transplanted intraportally in immunodeficient mice (14).

Hypothesis: IDN6556 will enhance marginal mass portal islet engraftment in a large animal islet autotransplantation model when administered orally

Materials and Methods

Animals

Adult female Yucatan miniature swine (22-32 kg) were obtained from the colony at Memorial University, St John, Newfoundland. Ethical approval was obtained from the animal welfare committee at the University of Alberta, and all animals were maintained and cared for according to the Canadian Council on Animal Care guidelines.

Caspase inhibitor

IDN6556 was obtained as a generous gift from Pfizer Inc. (New York, NY) in powder form and was stored at room temperature. Animals received either IDN6556 20mg/kg orally in Phosphate Buffered Saline (PBS) (n=7, treatment group) or PBS control (n=6, control group) twice daily for seven days following transplantation, beginning on the first postoperative day. There was random assignment to groups. For the *in vivo* portion of the studies, IDN6556 was only given orally, initially by gavage, then mixed with the solid diet. IDN6556 was not administered during islet culture in the *in vivo* studies, as islets were transplanted in unpurified state, shortly following completion of the isolation process.

Pancreatectomy and Islet Transplantation

Animals were fasted > 12 hours prior to surgery. After induction of anaesthesia, endotracheal intubation, antibiotic prophylaxis (cefazolin, Novopharm, Toronto, ON) (1g intramuscularly) and central venous line access, physiological monitoring and intravenous fluid support, the abdomen was entered through a midline laparotomy, and a harmonic scalpel (Ethicon, Markham, ON) used to provide meticulous dissection for a total pancreatectomy. The major pancreatic duct was cannulated using a 24G catheter with the pancreas *in situ* to facilitate pancreas distension during the digestion phase. Shortly following pancreatectomy, a 5g intravenous bolus of arginine-HCL was administered and serum samples collected at 0, 2, 3, 4, 5, 7 and 10 minutes to confirm absence of insulin secretion and complete removal of the entire β-cell mass. After islet isolation, an unpurified marginal mass of islets was gravity infused into a tributary of the portal vein through a 7Fr catheter. After appropriate titration, and from previous studies in our laboratory, we found a marginal islet mass of 1000-1500 IE/kg, to be the ideal mass to delineate differences between the control and treatment groups (15). A 70 U/kg bolus of heparin was administered intraportally with the islets. Before closure, the duodenum was checked for viability in all cases.

Post-transplant care

Central access and intravenous fluids were maintained for 48 hours post surgery, then pigs were fed a clear fluid diet for two days returned to standard pig chow

supplemented with pancreatic enzyme replacement (Pancrease-V, Bioniche Animal Health Canada Inc, Belleville, ON). Blood glucose was monitored each morning using a glucometer (One Touch Ultra, Johnson and Johnson) and Lantus insulin was titrated as needed to maintain blood glucose levels ≤ 11 mmol/L. Low molecular weight heparin was administered for one week post transplant (Lovenox 1.5 mg/kg subcutaneously daily; Sanofi Aventis, Laval, Que). Pigs were weighed twice weekly. Opioid and non-steroidal anti-inflammatory analgesia was provided with buprenorphine (Schering-Plough, Hertferdshire, UK) twice daily and meloxicam daily (Boehringer Ingelheim, Burlington, ON) for five days post surgery.

Islet isolation

The pancreas was syringe-distended intraductally with cold enzyme solution (Liberase DL; Roche Applied Science, Mannheim, Germany) through the previously placed catheter. Digestion of the pancreas was conducted at 37°C in a Ricordi chamber; the time dependent on free islets appearing in a 1mL withdrawn sample. The mean dilution time was 19.27 min ± 3.9. Samples were stained with dithizone (Sigma Aldrich, Oakville, ON) (1:10 in PBS) prior to viewing and were counted by normalizing to a standard islet size using islet equivalents (IE) of 150um. Islet purity was measured according to the relative proportions of dithizone-positive to negative tissue. Viability was assessed with SYTO green (SYTO-13, Molecular Probes, Eugene, OR) and ethidium bromide (Sigma

Aldrich, Oakville, ON). Cells which stained green were considered viable while ethidium bromide stained dead or damaged tissue (16, 17).

In vitro testing of islet toxicity with IDN6556 in glucose-stimulated insulin release assays

Triplicate aliquots containing 1000 IE were washed in glucose-free medium (CMRL supplemented with 10% FCS). The media was then replaced with either low glucose medium (CMRL containing 2.8mM D-glucose and supplemented with 10% FCS) or high glucose medium (CMRL containing 20 mM D-glucose supplemented with 10% FCS) and incubated for 1hr at 37°C, 5% CO_2. A second triplicate was washed and incubated in similar media with the addition of the caspase inhibitor IDN6556 (100uM). Aliquots from the supernatants were analyzed for porcine insulin content using a radioimmunoassay (Linco Research, St Charles, MO). In each instance, the fold stimulation was calculated by dividing the insulin values released from islets in high glucose medium by that of islets in low glucose medium run in parallel.

In vivo glucose potentiation of arginine-induced secretion (GPAIS)

GPAIS were performed one-week prior to transplantation (N=13, naïve group) and at one month post transplant. Animals were fasted overnight, placed under general anaesthesia, and central venous access secured. Protocols for GPAIS

are as described previously (15). Briefly, blood samples were drawn at 5, 10, 15, 30, 50 and 60 minutes, then a 5g bolus of arginine-HCL given, with further sampling at 62, 63, 64, 65, 67 and 70 minutes. Blood glucose was measured glucose oxidase in standard assay, and porcine insulin was analyzed simultaneously with a sensitive radioimmunoassay kit (Linco Research, St Charles, MO, limits of sensitivity 0.02-1.0 ng/ml insulin). The acute insulin release in response to arginine (AIR_{ARG}) was calculated from the three peak serum insulin levels at 2, 3, 4 and 5 minutes after arginine and subtracting the basal serum insulin level.

Statistics

Data was analyzed using GraphPad Prism (Version 5.0c, GraphPad Software Inc, San Diego, CA). P values less than 0.05 were considered statistically significant. Results are expressed as mean ± SEM. Kaplan meier curves were analyzed using log rank analysis and column means were compared using a t-test.

Results

IDN6556 substantially increases rates of euglycemia with marginal mass islet autotransplantation

Surgical complete pancreatectomy led to absence of stimulated insulin response in all treated animals (treatment and controls), confirming effective surgical resection of the beta cell mass (Table 4-1). There was no difference in the mean acute insulin release (AIR_{ARG}) immediately after pancreatectomy between the two groups. The cold ischemic time was short (approximately 30 minutes) and not different between groups (Table 4-1). The islet isolation was successful in all cases, with only a marginal mass returned to each pig (1000-1500 IE/kg). This equated to at least 49% of the islet mass (range 48-83%) being discarded, and this did not differ between groups. There was no difference in islet viability or islet score between groups (Table 4-1).

Figure 4-1A shows the mean daily fasting blood glucose values for the IDN6556 intervention and control groups; the marginal mass led to early hyperglycemia in both groups, but in the IDN6556 group, glycemic control improved markedly after day 12 (Day 16: mean blood glucose 11.02 vs 18.8, $p<0.05$; Day 23: mean blood glucose 8.4 vs 23.6, $p<0.05$). Figure 4-1B displays the dosage of insulin utilized each day, confirming that differences in fasting blood glucose were not due to disproportionate insulin usage. The proportion of pigs maintaining blood glucose <11mmol/L after marginal mass islet transplantation was significantly higher in

the IDN6556 treatment vs vehicle-treated control groups (Figure 4-1C; 100% IDN6556 vs 33.3% control, $p < 0.05$ by log-rank analysis).

Islet potency was not different between groups

Islet aliquots from the discarded portion of each isolation were assessed in triplicate for insulin release in response to glucose. Figure 4-2A demonstrates that there was no significant difference in *in vitro* potency of islets isolated from treatment group pigs vs control group pigs, based on the insulin stimulation index, (1.31 IDN6556 vs 1.21 control, $p > 0.05$, unpaired t-test). The stimulation index reflects the ratio of insulin release in islets exposed *in vitro* to low and high glucose.

IDN6556 is not toxic to islets *in vitro*, and does not alter insulin release

To determine any detrimental impact of the drug IDN6556 on islets *in* vitro, the stimulation index was further assessed in triplicate in control islets maintained in culture for two hours vs in the presence of 100uM IDN6556 (Figure 4-2B). There was no significant difference in the stimulation index between groups (1.18 IDN6556 vs 1.26 control, $p > 0.05$ by paired t-test).

IDN6556 treatment improves metabolic reserve in marginal mass islet transplants

One month after islet autotransplantation, pigs were fasted overnight and subjected to arginine potentiated glucose tolerance testing. In the IDN6556 treatment group, glucose tolerance did not differ significantly from the normal, naïve pigs (pre-operative controls, n=13), Figure 4-3A. This improved glucose tolerance translated into an improved area under the curve (AUC) when comparing IDN6556-treated and control (vehicle) pigs (Figure 4-3B, $p < 0.05$ by unpaired t-test). Similarly, the AUC did not differ between IDN6556 treatment and baseline control normal pigs, demonstrating that the marginal, engrafted islet mass, protected by IDN6556, was sufficient to provide normal metabolic reserve. In response to arginine potentiation (Figure 4-4), the AIR_{ARG} of the IDN6556 group was significantly higher than the vehicle control (0.30 ng/ml IDN6556 vs 0.16 ng/ml control, $p < 0.05$ by unpaired t-test).

IDN6556 did not lead to systemic toxicity in pigs

We compared body weight, activity and signs of toxicity between groups, and identified no evidence for systemic toxicity in the IDN6556 treated group, when given at a dose of 20mg/kg twice daily for 7 days. Formal toxicology studies were not conducted, as there is clinical data demonstrating safety and lack of toxicity. There was no mortality related to IDN6556 treatment. Terminal laparotomies at one month revealed no evidence of infection or malignancy.

Discussion

This study of unpurified, intraportal islet transplantation in a large animal, pig model confirms our previous findings and hypothesis that apoptosis remains a dominant target for islet loss in the early post transplant period, and that prevention of early apoptosis can substantially augment islet engraftment mass. We have previously investigated caspase inhibitors as a means of preventing islet apoptosis; zVAD-FMK and EP1013 led to enhancement of islet engraftment in mouse models of diabetes both using marginal mass syngeneic grafts and using human islets(18, 19). In marginal mass transplants, we were consistently able to reduce the mimimal islet implant mass to less than 30% compared to control transplants when different caspase inhibitors were given for up to 10 days post transplant in mice, when transplanted with syngeneic islets or human islets transplanted into immunodeficient mice (18, 19). The mechanism underlying this was predominantly reduced apoptosis as measured by Terminal Deoxy-nucleotide Transferase dUTP Nick End Labeling (TUNEL) observed at 24 hours post transplant.

The fluoromethylketone group in zVAD-FMK has potential toxicities and is unsuitable for clinical application; EP1013 is no longer accessible as the company Epicept folded. In seeking a potent pan-caspase inhibitor for potential clinical translation, we identified IDN6556 (3-[2-(2-tert-butyl-phenylaminooxalyl)-amino]-propionylamino]-4-oxo-5-(2,3,5,6-tetrafluoro-phenoxy)-pentanoic acid) as a more suitable compound, as it has already been tested as a liver-targeted

apoptosis inhibitor and used previously together with clinical immunosuppression (20). When tested in mice, the drug was well tolerated, was not associated with either detrimental islet toxicity or systemic toxicity, and the groups treated with IDN6556 demonstrated marked improvement in islet engraftment, with sustained function over time for prolonged periods after discontinuation of the agent (*14*). The aim of the current study was to further explore IDN6556 in a large animal, porcine marginal mass islet transplantation model. IDN6556 was administered orally in the present study to take potential advantage of first-pass portal adsorption and direct protection of the intraportal islet mass (21).

We confirmed that complete surgical pancreatectomy led to a diabetic state, with absent acute insulin release. We observed substantial improvement in the engraftment of marginal mass, unpurified pig islets with 7 day treatment with IDN6556, with improved glycemic control, and metabolic control that resembled the normal, naïve pig profile. A marginal mass of 1000 – 1500 IE/kg unpurified islets was sufficient to reverse diabetes in all cases (7/7) in the IDN6556 treatment group, but sufficient in only 2/6 in the control group. Approximately half of the isolated islet mass was discarded in both groups – therefore potentially one pancreas may have been sufficient to treat two recipients in the IDN6556 group (not tested in the autograft state).

Oral administration of IDN6556 therefore appears to be effective in first-pass intraportal adsorption and direct islet protection in this large animal model. In our

previous reports, we used intraperitoneal dosing in mice (*14, 18, 19*). It is possible that more prolonged or increased oral dosing may provide even further protection, but this was not tested in the present study.

In terms of mechanism of protection, we have consistently found previously that apoptosis was reduced with pan-caspase inhibitors, including IDN6556 in mouse and human islets transplanted into mice, based on TUNEL assays (*14*). However, we did not repeat liver biopsies at 24 hour time points in the present study, as this would have required more large animals, and repeated surgical interventions were not felt to be appropriate.

We identified no evidence of islet toxicity from systemic IDN6556 treatment, and similarly found no signs of islet toxicity *in vitro*. In terms of systemic toxicity, the current study was not designed to provide formal toxicology data, but specifically we found no associated mortality, morbidity or specific toxicity from IDN6556 at 20mg/kg twice daily in pigs. Terminal laparotomies revealed no signs of malignancy or infection, but clearly the time-frame for observation is limited. Clinical phase I/II dose-escalation and dosing of IDN6556 for up to 12 weeks in subjects with underlying hepatitis C failed to demonstrate increased rates of malignancy or toxicity, and led to improved rates of hepatocellular protection in the presence of hepatitis C (*19, 20*). Furthermore, there appeared to be no synergistic toxicity when IDN6556 was combined with standard immunosuppression in patients undergoing liver transplantation, when IDN6556

was added both to the preservation flush solution and to subjects at a dose of 0.5mg/kg intravenously for 24 hours post transplant, and the compound reduced apoptotic hepatocellular injury (20). Prior studies had shown clear evidence of hepatoprotection in rodents (*20, 23-25*).

Limitations of the present study include: a) small numbers of large animals (limited based on cost, capacity for large animal housing and ethical considerations); b) the use of unpurified islets, making the accuracy of islet mass quantification less precise, due to transplantation of islet fragments or islets 'cuffed' in exocrine tissue; and c) the observation period being limited to one month post transplant. Therefore, while the present findings are encouraging, it remains to be seen whether similar positive findings will be replicated in clinical patients treated with IDN6556. Clinical studies are presently in development to test the impact of this compound in patients with type 1 diabetes receiving purified intraportal islet transplants.

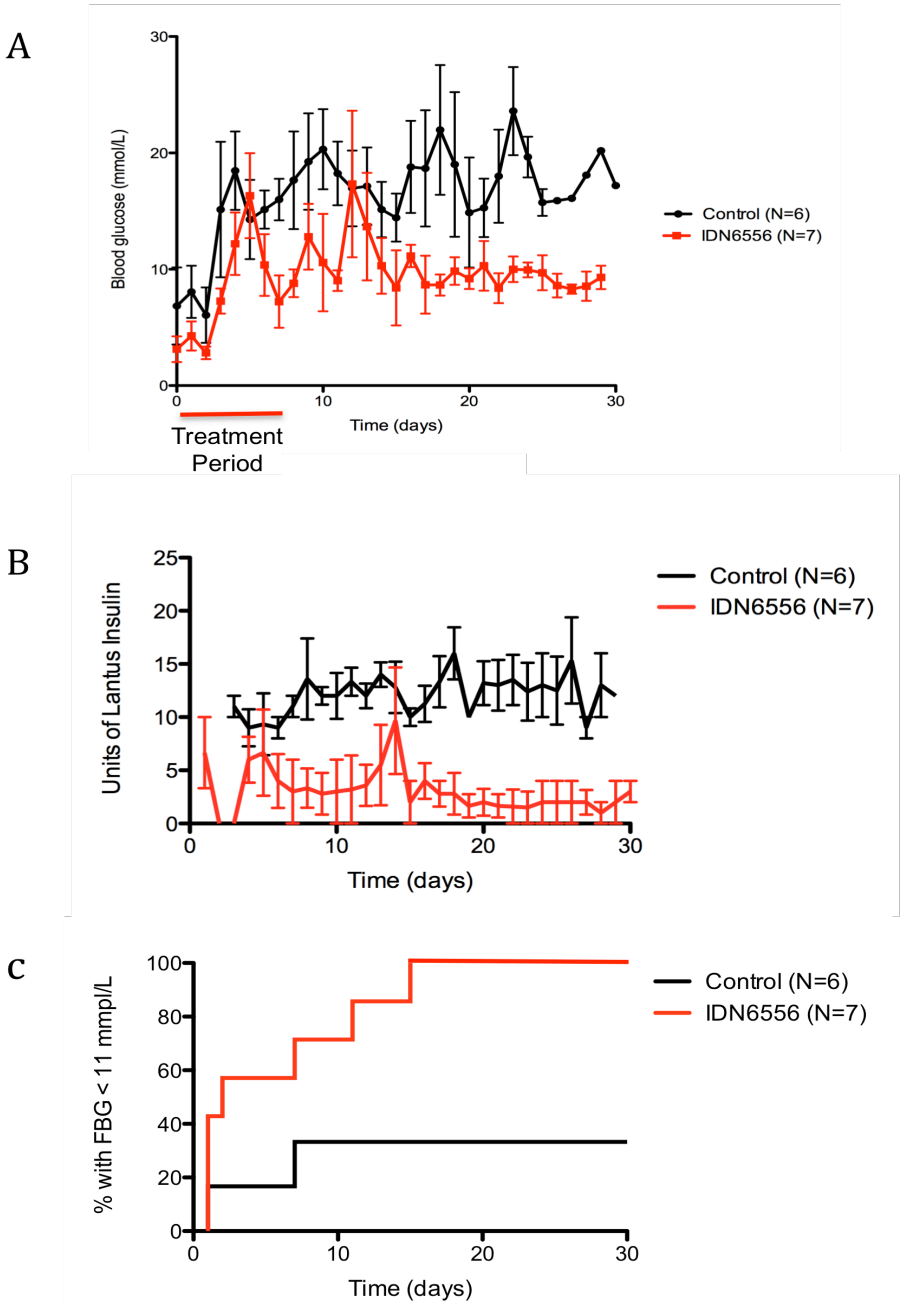
In summary, the pan-caspase inhibitor, IDN6556 improved marginal mass intraportal islet engraftment in a pig islet autotransplant model, and combined with the previous similar findings in mouse and human islets transplanted in mice, now provides compelling support for further investigation in the clinic.

Table 4-1 Comparison of pancreatectomy and isolations

	Control (N=6)	IDN6556 (N=7)	p-value
Pig weight[a] (kg)	26.43	23.71	0.35
Pancreas cold ischemic time (mins)	32.86	29.57	0.67
Islet mass isolated per kg body weight (IE/kg)	4112	3749	0.58
Islet mass isolated per gram pancreas weight (IE/g)	2833	2741	0.87
Islet viability (%)	91.2	92.3	0.71
Islet score[b]	5.9	5.9	0.90
AIR_{ARG}[c] post-pancreatectomy (ng/ml)	0.036	0.022	0.51
Average Islet mass infused (IE/kg)	1167	1214	0.75

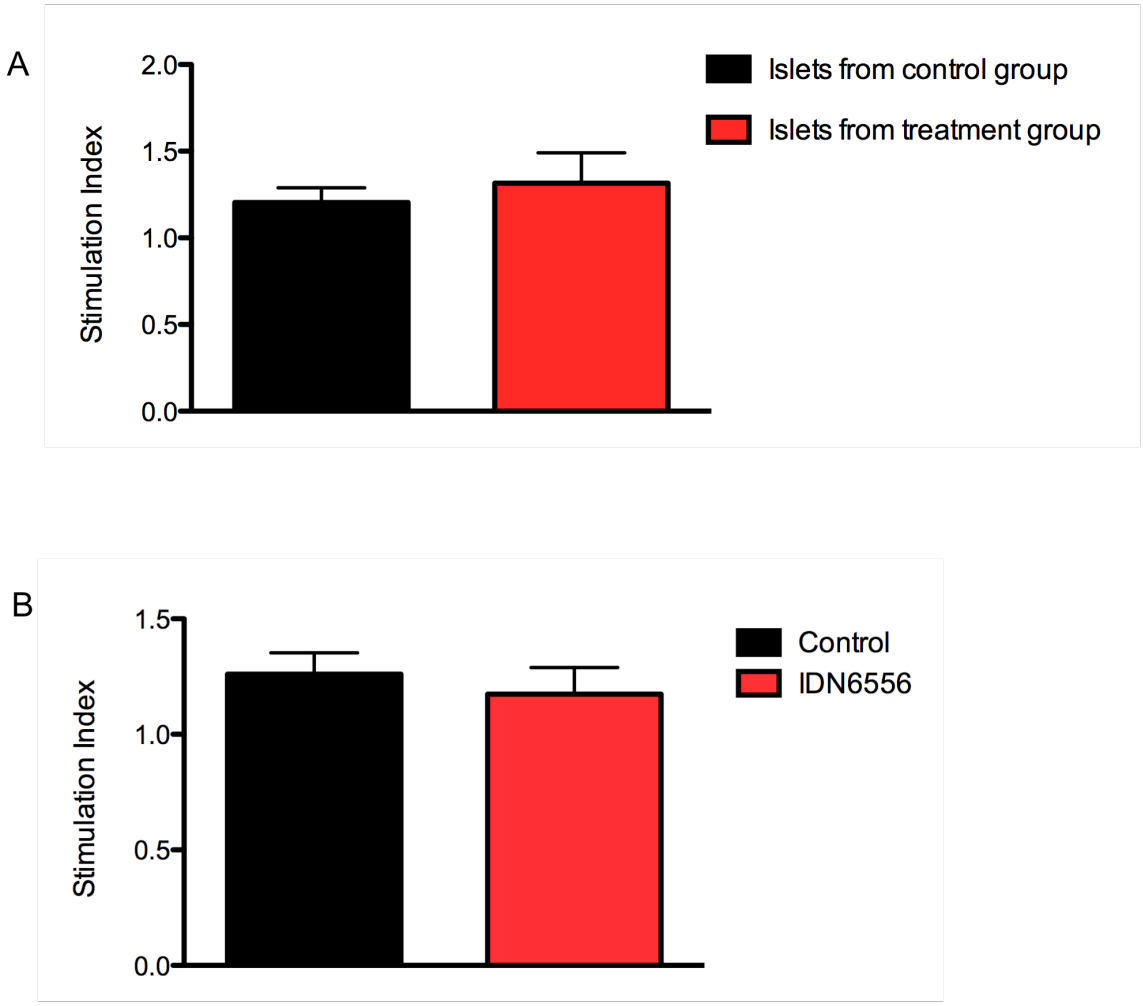
[a] At time of pancreatectomy
[b] A summation of scores of islet fragmentation, shape, density and uniformity
[c] Acute insulin release in response to arginine

Figure 4-1 Fasting blood glucose and daily insulin use



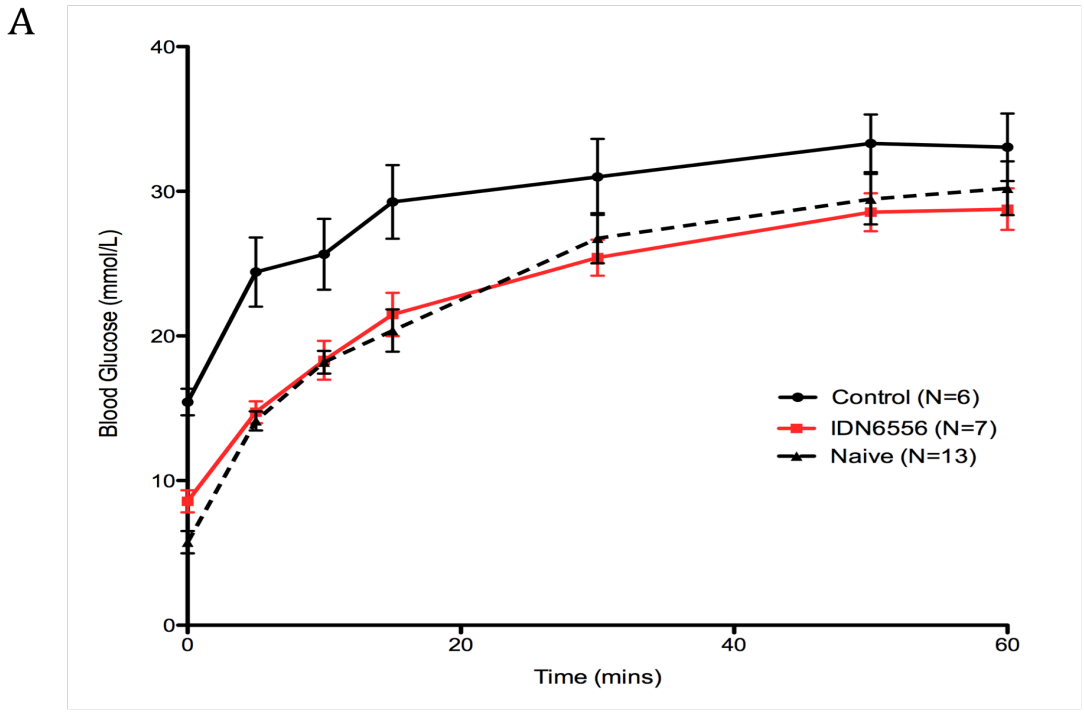
Female Yucatan swine underwent total pancreatectomy and marginal mass islet autotransplantation. For one week post-transplant, pigs were treated with either the caspase inhibitor IDN6556 (20 mg/kg orally bid, N=7) or vehicle (PBS, N=6). Fasting blood glucose values were collected daily (A). The total average daily dose of Lantus insulin given is displayed in B. Figure C displays the percentage of pigs with persistent blood glucose <11 mmol/L as a function of time after transplantation ($p<0.05$ by log-rank analysis)

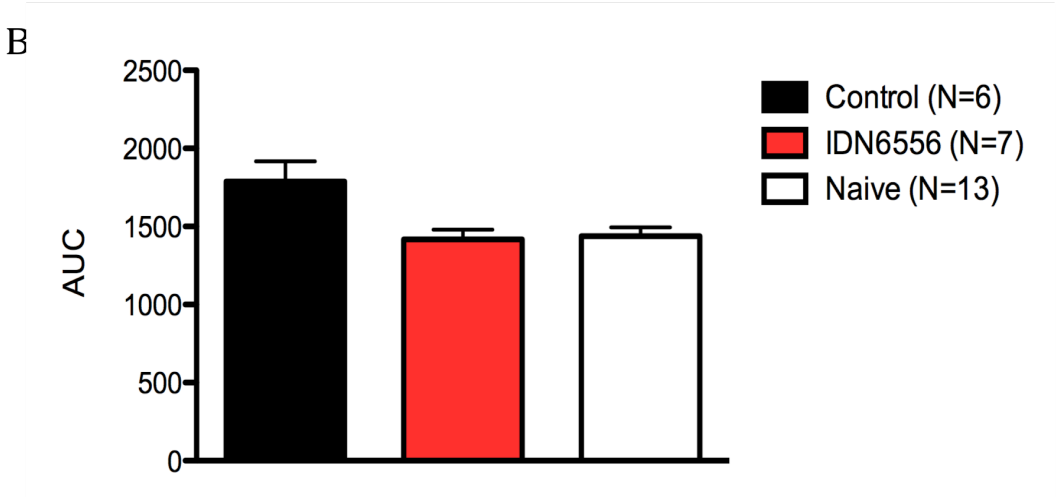
Figure 4-2 In vitro assessment of insulin secretory function of isolated islets



There was no significant difference in stimulation index between islets isolated from IDN6556-treated pigs and those isolated from vehicle-treated pigs (A) ($p > 0.05$ by t-test). For each islet isolation, separate triplicate aliquots were set aside and treated as above except for the addition of IDN6556 to all media. There was no difference in stimulation index between islets incubated in the caspase inhibitor and those without ($p > 0.05$ by t-test) (B).

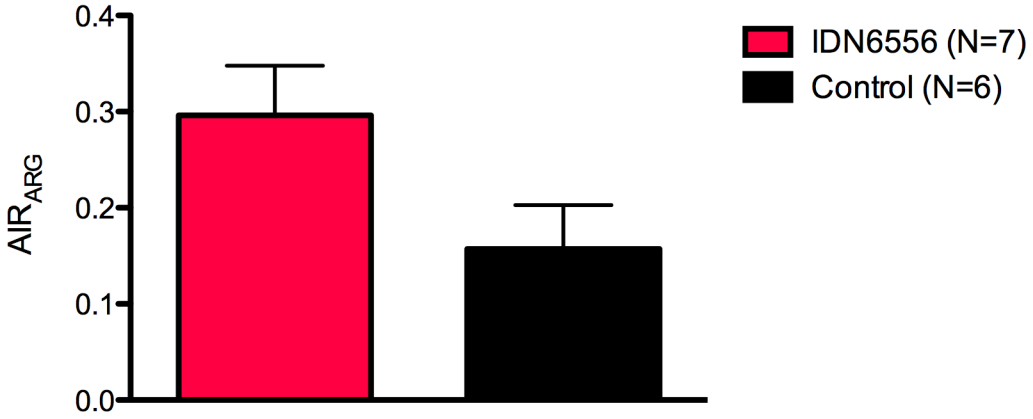
Figure 4-3 Glucose tolerance one month following islet transplantation





Pigs were fasted overnight and given a 500cc intravenous infusion of 20% dextrose over one hour. Blood glucose was sampled throughout the hour (A). All pigs underwent a similar procedure one week prior to pancreatectomy (naïve). Area under the curve (AUC) values for theses curves are displayed in B. Pigs treated with IDN6556 for one week post-transplantation displayed improved glucose tolerance as compared to vehicle-treated pigs ($p < 0.05$ for AUC values). There was no difference between the glucose tolerance of naïve and IDN6556-treated pigs one month after marginal mass islet transplantation ($p>0.05$).

Figure 4-4 Acute insulin release in response to arginine (AIR_{ARG})



At one month, immediately after the dextrose infusion/glucose tolerance test, a 5g bolus of arginine was administered intravenously. Serum was sampled over the following ten minutes to determine porcine insulin release. Pigs treated with the caspase inhibitor IDN6556 displayed a significantly higher AIR_{ARG} as compared to vehicle-treated pigs ($p < 0.05$ by t-test).

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Chapter 5

Preface to Chapter 5

A further stress on islets is the cytokine milieu encountered immediately after transplantation. This includes **IL-1** and **TNF-alpha**. Infliximab and etanercept (more recently) are being utilized in islet peri-transplant cocktails to enhance outcomes. In fact, etanercept has been linked to the recent success of single donor islet transplants in some centers. This current clinical use is based on **one early pre-clinical mouse study** using mouse, not human, islets and does not have randomized trial support (very difficult with low islet transplant numbers). In fact a recent series used etanrecept in combination with IL-1 receptor antagonism (anakinra) and showed benefit to engraftment at one month, although the study was underpowered (n=3 transplants).

The purpose of experiments described in Chapter 5 is therefore to **study the effects of etanercept with or without anakinra on engraftment of both mouse and human islets (marginal mass islet grafts)**. The **metabolic reserve** and **islet survival** will be assessed at one month with a look into possible **mechanisms** for their effects.

The combination of an Interleukin-1 receptor antagonist and tumor necrosis factor-α blockade improves marginal mass engraftment of human islets in immunodeficient mice

A version of this chapter has been accepted for publication in the *American Journal of Transplantation*

Introduction

Inflammatory injury of transplanted human islets is recognized by most clinical islet programs as a major remediable target, as judged by the large proportion of clinical centres that routinely and empirically administer anti-inflammatory treatments post transplant. Since the early days of islet transplantation, anti-oxidants (pentoxiphylline, multivitamins), anti-inflammatories (aspirin, non-steroidal anti-inflammatory agents), and relatively expensive and specific anti-tumor necrosis factor-alpha (anti-TNF alpha) blockade with infliximab or more recently etanercept, has been a keystone component of peritransplant management of at least 80% of islet transplants performed, according to the Clinical Islet Transplant Registry (CITR) summary reports (1). The routine use of anti-TNF strategies has been based solely on one preliminary report in a syngenic mouse islet transplant model, but without formal supportive data in a more relevant human islet transplant model (2). The routine clinical adoption of anti-TNF-alpha strategies is therefore nearly empiric, and lacks supportive data from randomized controlled trials. Hering et al. reported high rates of single donor islet transplant engraftment and insulin independence, with etanercept being a major component of the regimen (3, 4). Most recently, one centre has advocated the combined use of TNF alpha blockade together with an IL-1 receptor antagonist (IL-1ra), but with only three patients treated, the results are encouraging but difficult to interpret due to limited power (5). We therefore set out to investigate the potential synergy of a soluble dimeric TNF-alpha receptor

fusion protein (etanercept) when used alone and in combination with an IL-1Ra (anakinra), when given to chemically diabetic immunodeficient mice receiving marginal mass human islets. The purpose of this study is therefore to provide additional preclinical data to determine the appropriate utility of these agents in clinical islet transplantation.

Hypothesis: The combination of anakinra and etanercept will provide the most pronounced benefit to islet engraftment as compared to either agent alone

Materials and Methods

Animals and Reagents.

Immunodeficient B6-RAG $^{-/-}$ mice (B6.129S7-*Rag1tm1Mom*/J) were obtained from the Jackson Laboratories (Bar Harbor, ME) and housed under specific pathogen-free conditions. BALB/c mice were also obtained from the Jackson Laboratories but housed under conventional conditions. All animals were cared for according to the guidelines of the Canadian Council on Animal Care, and ethical approval was obtained from the animal welfare committee at the University of Alberta. All reagents were obtained from Sigma Aldrich (Oakville, Ontario, Canada) unless otherwise specified. Anakinra was purchased from Biovitrum (Stockholm, Sweden). Etanercept (Amgen Inc, Thousand Oaks, CA) was purchased commercially from the University of Alberta hospital pharmacy.

Mouse Islet Isolation.

Mouse islets were isolated using established protocols with minor modifications (6). In brief, mouse pancreata were digested with collagenase (1.0mg/ml in Hanks' buffered saline solution (HBSS)) and purified with Histopaque-density centrifugation (Sigma Aldrich, Oakville, ON). Handpicked islets were washed with HBSS then placed in short-term culture in Connaught Medical Research Laboratories (CMRL-1066) medium supplemented with 10% fetal bovine serum, L-glutamine (100 mg/l), penicillin (112 kU/l), streptomycin (112 mg/l) and HEPES

(25 mmol/l). Islets were cultured for a maximum of two hours before transplantation.

Human Islet Isolation.

Pancreata were retrieved from multiorgan deceased donors after aortic cross-clamp and infusion of Histidine-Tryptophan-Ketoglutarate (HTK) solution. Islets were isolated according to a modified Ricordi's semi-automated technique(7, 8). Briefly, the pancreas was distended with collagenase NB1 supplemented with neutral protease (Serva Electrophoresis GMbH) and digested in a Ricordi chamber. When free islets were released, tissue digest was collected and further purified on a cell sorter (Model 2991, Cobe, Lakewood, CO) using a continuous density gradient(9). Human islets were processed in all cases with intent for clinical transplantation, but made available for this study when the islet yield fell short of the minimal mass required for clinical transplantation, and where specific research consent allowed for use of human islets. Permission to use human islets for these studies was granted by the Health Research Ethics Board of the University of Alberta. Human islets were cultured overnight in CMRL-1066 supplemented with insulin-selenium-transferrin and insulin-like growth factor-2 (IGF-2) at 37°C before transfer to the laboratory and transplantation into diabetic mouse recipients.

Islet Transplantation.

Streptozotocin was administered to recipient mice to induce diabetes (BALB/c: 220mg/kg intraperitoneally (i.p.); B6-RAG$^{-/-}$: 180mg/kg i.p). Animals were considered diabetic after two consecutive blood glucose measurements ≥ 20 mmol/L using a OneTouch Ultra glucometer (Lifescan Canada, Burnaby, B.C). For mouse islet studies, a marginal mass of 150 islets were implanted into the kidney subcapsular space. In human islet studies, an equivalent marginal mass of 1,500 human islet equivalents (IE) were implanted beneath the kidney capsule, based on our previous and ongoing experience with marginal mass islet transplantation of human islets in this mouse model (10)Transplant recipients were divided into four groups and treated with: 1) anakinra 100 mg/kg i.p. daily for 7 days (n=12 for human islets, n=11 for mouse islets); 2) etanercept 5 mg/kg i.p. on days 0, 3, 7 and 10 (n=11 for human islets, n=16 for mouse islets) to mimic the current clinical dosing schedule introduced by Hering et al., and now adopted by several islet transplant programs worldwide (3, 11); 3) IgG (control antibody) 5 mg/kg i.p. on days 0, 3, 7 and 10 (n=11 for human islets, n=11 for mouse islets); 4) Combined treatment with anakinra and etanercept at the doses above (n=16 for human islets, n=13 for mouse islets). Blood glucose of recipients was monitored daily by tail-vein glucometer readings.

Glucose tolerance tests.

Transplanted mice were fasted for 16-20 hours and injected intraperitoneally with 50% dextrose at 2g/kg body weight (intraperitoneal glucose tolerance test, IPGTT). Blood glucose levels were analyzed at baseline, 5, 15, 30, 60, 90 and 120 minutes post-injection

Graft insulin content.

Islet grafts were harvested from the kidney capsule and stored at -80°C for bulk analysis. Extraction was performed in acid-ethanol by homogenization and ultrasonic cell membrane disruption. Insulin concentration of the neutralized extract was measured using a commercial ELISA kit (Alpco Diagnostics, Windham, NH).

Human Islet Culture and viability.

A portion of each human islet isolation (n=4 islet preparations) was separated into four groups and placed in culture in CMRL-1066 at 37°C. The media was supplemented with: 1) anakinra 10 ug/ml; 2) etanercept 100uM; 3) IgG-control; or 4) anakinra and etanercept. Islets were counted using dithizone at the beginning of culture and at the 48 hour point. Viability was assessed using SYTO green/ethidium bromide, counting 100 islets under fluorescence light microscopy as previously described (Cedarlane laboratories and Sigma-Aldrich, ON, Canada) (12, 13).

Apoptosis Assays

Apoptosis of islet cells within transplanted grafts was quantified using TUNEL staining (Dead-end Apoptosis Detection System, Promega, Madison, WI) Nuclear counterstaining with DAPI (Molecular Probes,
Eugene, OR) was used to detect all cells present in the sample. Islet grafts were harvested, placed in formalin, processed and embedded in paraffin. To quantify apoptosis in vivo, fields containing at least 500 cells were analyzed at 200x magnification. The number of TUNEL+ cells (green) within the insulin+ islet graft area of the section were counted and compared to the total number of DAPI+nuclei within that same field to determine % apoptosis. Sections were prepared from 3 transplant recipients in each cohort, and at least 3 fields were analyzed in each section.

Results

Combined etanercept and anakinra substantially improves marginal mass human islet engraftment in immunodeficient mice

Immunodeficient, diabetic B6-Rag $^{-/-}$ mice received a marginal mass human islet graft (1,500 IE) under the kidney capsule and one of the four treatments defined above. Those receiving anakinra and etanercept had the highest rate of diabetes reversal (87.5%) (two consecutive non-fasting blood glucose readings < 11mmol/L), which was significantly higher ($p<0.05$ by log-rank analysis) than mice receiving anakinra (53.8%), etanercept (45.5%) or IgG control (36.4%) (Figure 5-1). The use of etanercept or anakinra as single agents failed to significantly enhance marginal mass human islet engraftment in this model. Twenty-four hours after transplantation, mice were sacrificed and grafts assessed for apoptotic cells (n=3 per group, processed in triplicate) (Figure 5-2). Grafts from mice in the control group had 23.47% apoptotic beta cells within the graft, which was significantly more than mice treated with anakinra (7.85%), etanercept (12.44%) or combined anakinra and etanercept-treated mice (0.67%) ($p<0.001$, ANOVA). The combination group (anakinra + etanercept) demonstrated significantly lower islet cell apoptosis than the etanercept group ($p<0.05$, bonferroni post-hoc analysis).

After one month, all transplanted immundeficient mice were fasted overnight and underwent an IPGTT (Figure 5-3A). Area under the curve analysis for the IPGTT

is displayed in figure 5-3B. Mice receiving anakinra and etanercept displayed significantly improved ($p<0.05$) glucose tolerance when compared to control mice. Forty-eight hours later, mice were sacrificed and grafts recovered for insulin content analysis. Mice receiving combined anakinra and etanercept had significantly higher mean insulin content in their human islet grafts (10,874 mIU/ml) as compared to mice receiving anakinra (171,024 mIU/ml), etanercept (178,215 mIU/ml) or IgG control (119,614 mIU/ml) ($p<0.05$, ANOVA).

Anakinra and etanercept are not toxic to human islets in vitro

Four separate human islet preparations were cultured for 48 hours at 37°C in CMRL supplemented with: 1)anakinra; 2) etanercept; 3) IgG2 isotype-specific control; and 4) anakinra and etanercept At 24 hours of culture, there was no difference in the percentage of islet equivalents remaining (Figure 5-4A) (anakinra: 73.6%, etanercept: 75.2%, anakinra & etanercept: 82.7%, control: 76.8%; $p>0.05$ by ANOVA). There was also no difference in the viability of islets at 24 hours obtained with the SYTO green/EtBr technique (anakinra: 62.57%, etanercept: 72.43%, anakinra & etanercept: 72.43%, control: 63.97%; $p>0.05$ by ANOVA). After a further 24 hours there was no significant difference in islets remaining or viability between the groups (Figure 5-4B) (anakinra: 55.36% IE remaining, 80.8% viability; etanercept: 51.29% IE remaining, 75% viability; A&E: 66.13% IE remaining, 81.4% viability; control: 39.75 IE remaining, 70.4% viability $p>0.05$ for both IE remaining and viability by ANOVA).

Combined anakinra and etanercept improves marginal mass islet engraftment in syngeneic mouse islet transplantation

To further test the impact of these anti-inflammatory agents in a syngeneic mouse marginal mass model, we found that the combination of anakinra and etanercept displayed the highest rate of diabetes reversal (61.5%) followed by those receiving etanercept alone (31.8%), control (27.3%) and the anakinra group (18.2%) ($p<0.05$ for anakinra and etanercept vs anakinra by log-rank analysis) (Figure 5-5). Administration of either etanercept or anakinra given alone however failed to demonstrate significant benefit over control.

After one month, recipient syngeneic mice were fasted overnight and underwent glucose tolerance testing (Figure 5-6A). Area under the curve (AUC) was calculated (Figure 5-6B) for each curve in Figure 6A. There was no significant difference in AUC between non-hyperglycemic mice in each of the groups ($P>0.05$ by one-way ANOVA). Forty-eight hours after glucose tolerance testing, all mice were sacrificed and their graft analyzed for insulin content (Figure 5-6C). There was no significant difference between the groups (anakinra 151.1 ng/ml, etanercept 131.2 ng/ml, anakinra and etanercept 186 ng/ml, control 103.3 ng/ml; $p>0.05$ by ANOVA)

Discussion

The use of cocktails of different anti-inflammatory agents has become integrated into the routine peritransplant management of clinical islet transplant patients, often without robust supportive appropriate preclinical or clinical data, and with substantial cost of therapy. The justification for this empiric approach has been based on: 1) prohibitive cost of randomized controlled trials, 2) lack of adequate power based on limited numbers of clinical islet transplants, and 3) temptation of centres to adopt any potential therapy that might have positive impact in clinical islet transplantation, irrespective of the presence or absence of supportive data. It is estimated that approximately 700 subjects have received islet transplants worldwide. By contrast, the phase III trials required to justify safety and efficacy of anti-TNF-alpha and IL1Ra in rheumatoid arthritis involved large numbers of patients per arm, clearly not feasible in islet transplantation given the relative shortage of donor transplant organs (14-16).

The application of TNF-alpha blockade became integrated into clinical islet transplantation largely based on a single publication by Farney et al. employing a soluble TNF receptor in syngeneic mouse islet and not human islet transplants in mice (2). Hering et al. promoted the clinical use of etanercept in their single-donor islet transplant series(3) as part of several innovations both in islet preparation and in peritransplant management (17). Oberholzer et al. also adopted routine use of etanercept in their single donor transplant series (11).In

Edmonton, we initiated a randomized controlled trial for the investigation of an alternative anti-TNF-alpha medication (infliximab 10mg per kg per day for 14 days), but abandoned this trial after the first 12 subjects were treated, as we found no significant difference in rates of single-donor transplant success, and recognized that a large number of subjects would be required to detect a small difference in outcome (Shapiro et al., unpublished data). The Miami group also investigated infliximab in clinical islet transplantation, and found no significant clinical benefit (18).

The agent IL1Ra (anakinra) has also been found to be highly effective in controlling inflammation in rheumatoid arthritis in over 2,000 subjects, as reviewed in a recent comprehensive Cochrane Review (19). Larsen et al. found improved glycated hemoglobin and enhanced C-peptide secretion in patients with Type 2 diabetes (20). Matsumoto et al. adopted the combined strategy of anakinra and etanercept in three subjects undergoing clinical islet transplantation, and while the early results were promising, they were unable to sustain this trial through lack of funding. Of concern, Health Canada and the Food and Drug Administration issued a health warning regarding the chronic co-administration of these two agents in patients with rheumatoid arthritis, as with 24-week therapy there was a higher incidence (7%) of serious infections compared with monotherapy (1.8%) (21, 22) .Clearly the short-term, one-week administration of anakinra and etanercept in the islet transplant setting is different from the chronic dosing required in rheumatoid arthritis, but islet transplant

patients are also subjected to profound T-cell depletional inductional and potent immunosuppressive maintenance therapies, thus this potential risk does merit consideration.

We therefore set out to revisit the potency of both anti-TNF-alpha and IL1Ra therapies in a preclinical model of human islet transplantation using the marginal mass approach in immunodeficient mice, principally to assess efficacy in a stringent, relevant, islet engraftment model.

The principal findings of the current study are that the combination of both anakinra and etanercept led to marked and meaningful improvement in the engraftment of both human and mouse islet transplants in mice. Each of these agents when used alone failed to produce statistical significance, but synergy in response to the combination of therapies led to positive impact, supporting the approach of Matsumoto et al.

IL-1, TNF-alpha and interferon-gamma are generally regarded as the most toxic cytokines mediating islet injury in laboratory models (23-28). IL-1 causes detrimental effects on pancreatic islets including decreased insulin secretion, and islet death (26). The binding of IL-1 to its receptor (IL-1R) leads to the activation of NF-κB and the regulation of multiple genes including IL-1, IL-6 and TNFα (29, 30). Activation of NF-κB also leads to the expression of inducible nitric oxide synthase (iNOS) and the subsequent production of nitric oxide (NO), a potent cause of β-cell apoptosis (25, 26, 31). TNFα can kill β-cells directly through

binding to surface receptors, which contain a death domain, and works together with IL-1 to stimulate NO production(32). Preventing the action of these cytokines on transplanted islets has therefore been hypothesized to protect the engrafting β-cell mass in the early post-transplant period, where over 60% of the infused islet mass is destroyed within hours to days post transplant (33, 34).

To better understand the underlying mechanisms associated with the positive improvement in islet engraftment in the current study, we found that the dominant protective effect was mediated through a significant reduction in early (24hr) post-transplant apoptosis when mice are treated with either anakinra or etanercept, with the combination producing a more profound effect, leading to improved 30-day metabolic reserve. Clearly there could be several other mediators favoring islet survival in our study that may be unrelated to the effects upon apoptosis.

Binding of IL-1 to its receptor leads to intracellular changes which can negatively affect β-cells, including the activation of, MAPKs (ERK, p38, JNK), NF-κB, protein kinase Cδ and the induction of Fas expression(35-38). Activation of the former three lead to iNOS expression and subsequent β-cell apoptosis while the latter increases cell sensitivity to Fas ligand (FasL) and downstream caspase activation. TNFα binds to its receptor, which contains an intracellular death domain, leading to downstream Nf-κB activation and direct activation of the caspase cascade(39). In addition, TNFα strongly potentiates the cytotoxic effects of IL-1 on β-cells by synergistically augmenting the MAPK signaling pathway(40).

This may explain the synergistic effect of the combination of IL-1 and TNFα blockade in this study.

We did not observe any detrimental effect on islet survival when human islets were exposed to relevant concentrations of anakinra or etanercept *in vitro*, but in these studies we did not specifically challenge the human islets *in vitro* with addition of toxic cytokine cocktails (2, 23, 41, 42). We did not anticipate a protective effect therefore in our *in vitro* studies, however, there was not a detrimental effect on islet viability or survival.

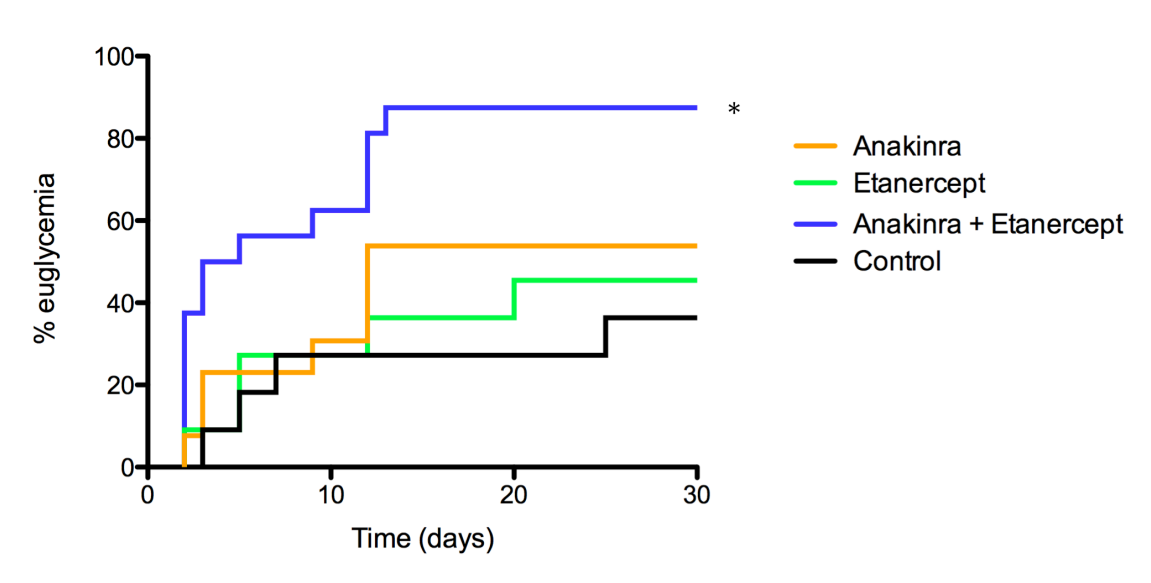
Interestingly, we found more positive impact of combined anakinra and etanercept in human islets than in the syngeneic mouse islet transplants. The exact reason for this is unclear, but likely reflects the fact that human islets have undergone much more prolonged periods of injury during brain death procurement, cold ischemic transportation, more prolonged isolation, purification and culture, than in the simpler mouse islet preparation model (7, 43-48). While the results of this study are promising, there are evident limitations that preclude direct extrapolation to the clinical setting. We did not directly quantify the cytokine milieu in the peritransplant site or in the serum of the transplanted mice, which may have revealed additional data.

We utilized the renal subcapsular space and not the intraportal site in this murine model, to facilitate graft explantation, insulin content and apoptosis, which would

have been more challenging in the intrahepatic site. Clearly the intraportal site may be associated with a different profile of dynamic cytokine exposure compared with the renal subcapsular space, that may have either masked or enhanced potential differences in therapeutic efficacy, but this was not explored further.

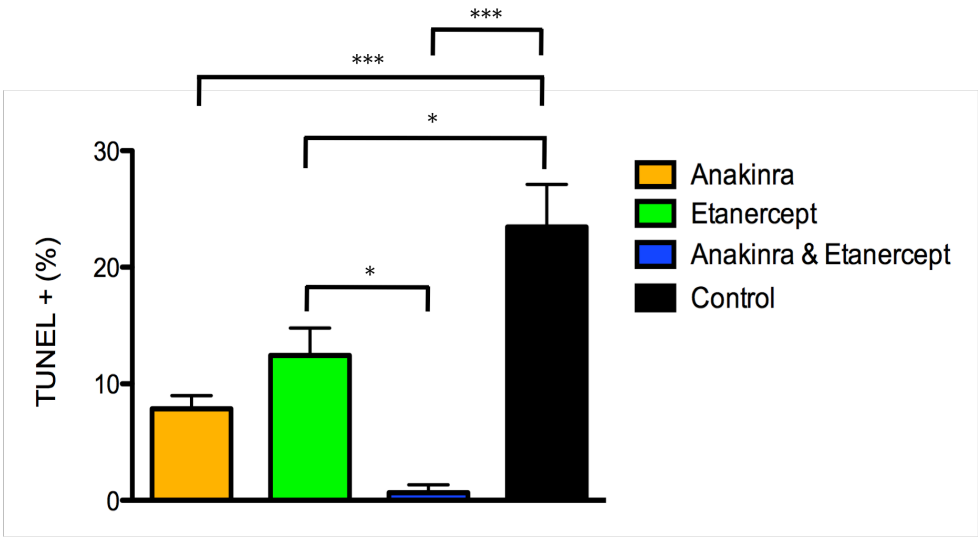
In summary, the current study provides important, positive supportive data to justify the combined use of both TNF-alpha blockade and IL1Ra therapy for short-term use in clinical islet transplantation. Conversely, the study questions the efficacy of etanercept monotherapy as currently adopted in most clinical islet transplant programs. Clearly, randomized controlled clinical trials are needed to fully address this issue, but will be challenging if not impossible to implement in the present environment of limited funding and limited clinical transplant activity.

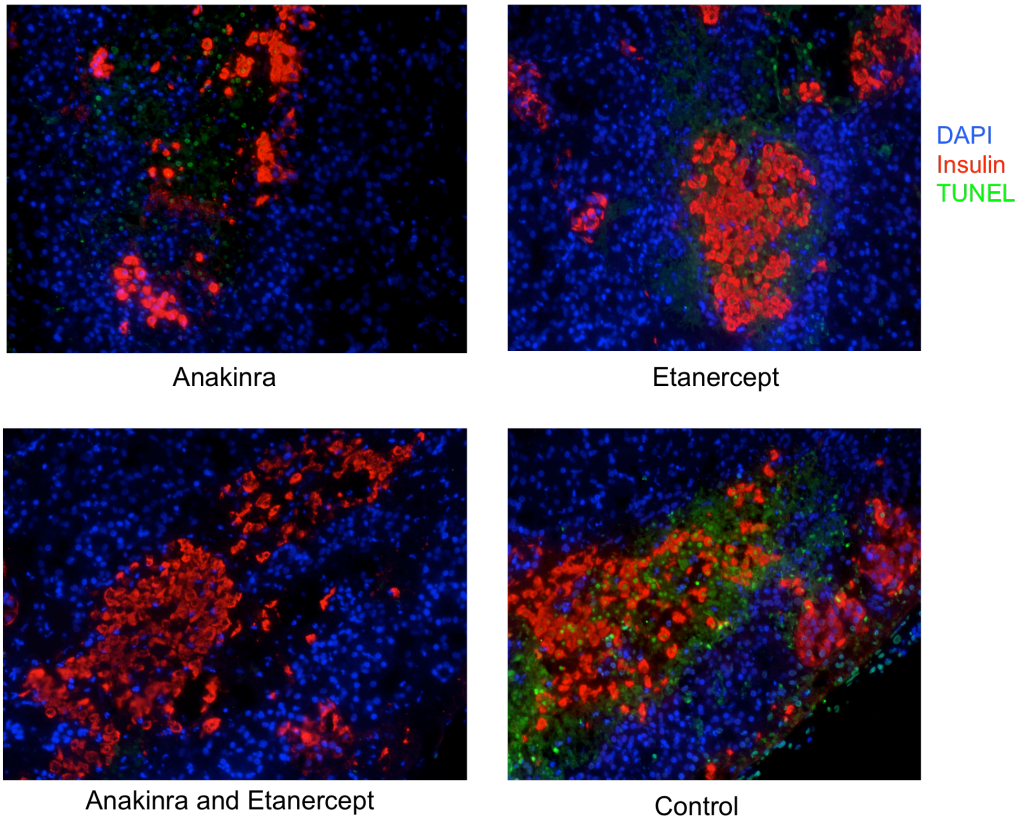
Figure 5-1 Diabetes reversal in mice receiving marginal mass human islet grafts and anti-inflammatory therapy



Immunodeficient mice received a marginal mass human islet graft (1500 IE) and either anakinra (N=12), etanercept (N=11), anakinra and etanercept (N=16) or IgG control (N=11). Euglycemia was defined as two consecutive non-fasting blood glucose readings < 11mmol/L. * $p<0.05$ for anakinra and etanercept vs all other groups.

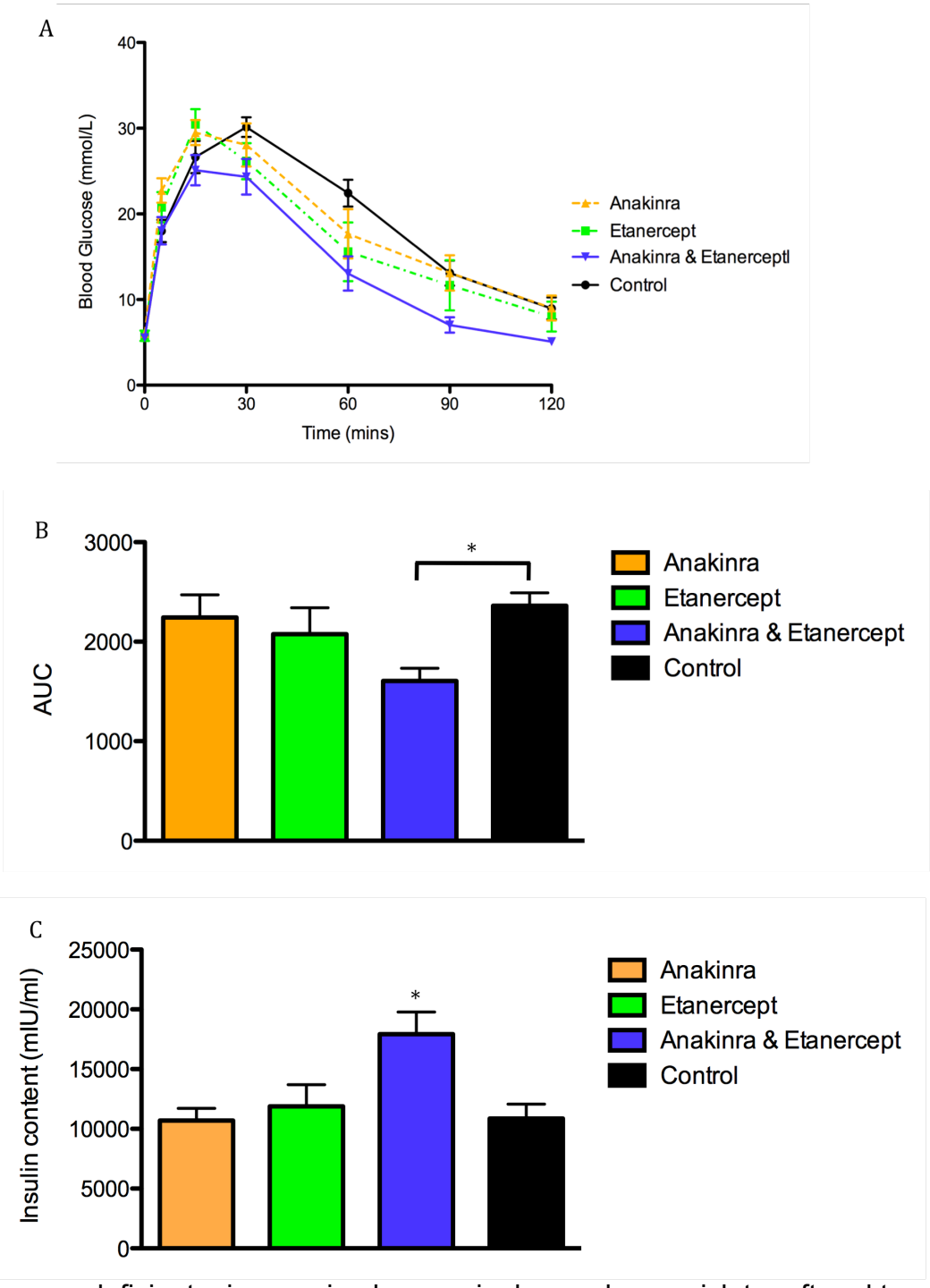
Figure 5-2 Apoptotic cells in human islet grafts





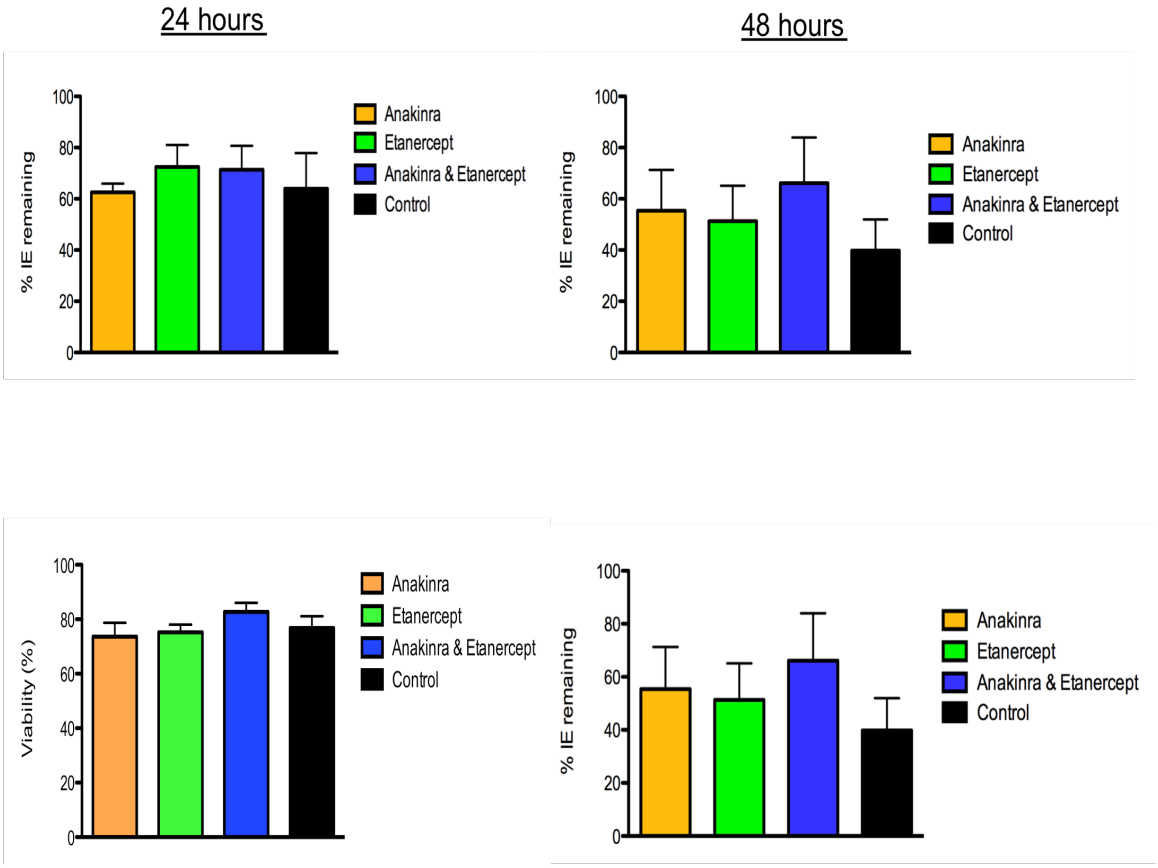
Twenty-four hours after transplantation, grafts were recovered from N=3 mice per group and assessed for apoptotic cells within the islet graft using TUNEL staining. Representative figures are displayed in B. * $p < 0.05$, *** $p < 0.001$.

Figure 5-3 Glucose tolerance and graft survival of human islets



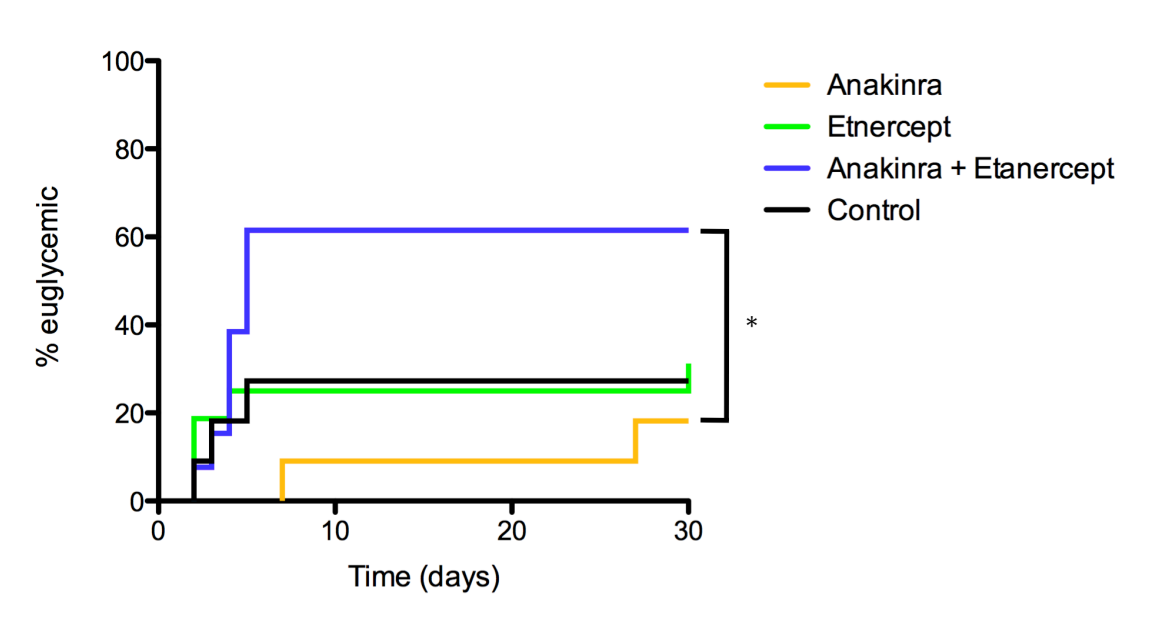
Immunodeficient mice received a marginal mass human islet graft and treated in one of four groups. After one month, glucose tolerance was assessed using an IPGTT (A). Area under the curve analysis is displayed in B. Forty-eight hours after the IPGTT, grafts were recovered and analyzed for insulin content (C). * $p < 0.05$.

Figure 5-4 In vitro human islet survival and viability



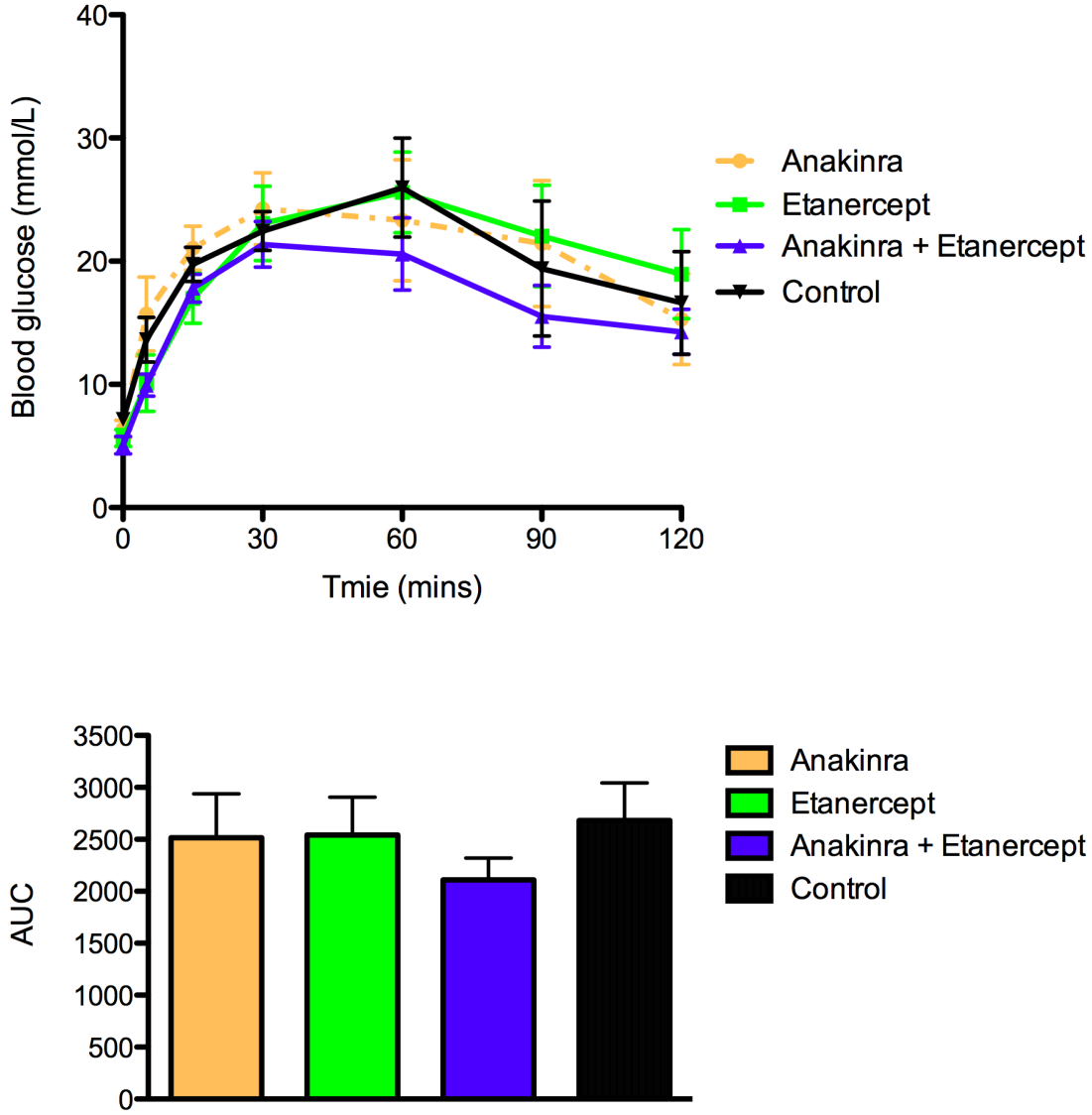
Human islets (n=4 preparations) were cultured in supplemented CMRL with anakinra, etanercept, a combination of anakinra and etanercept or IgG control. After 24 and 48 hours, islets were counted (islet equivalents, IE) and viability was assessed. $p>0.05$ by one-way ANOVA for all graphs.

Figure 5-5 Effect of treatment regimens on diabetes reversal after syngeneic marginal mass islet graft



Recipients were treated with anakinra (N=11), Etanercept (N=16), Anakinra and Etanercept (N=13) or IgG control (N=11). Diabetes was considered reversed with two consecutive blood glucose readings <11 mmol/L. * $p < 0.05$.

Figure 5-6 Glucose tolerance testing and graft insulin content analysis after one month



Syngeneic marginal mass islet graft recipients with non-fasting blood glucose < 18 mmol/L were fasted overnight and underwent an intraperitoneal glucose tolerance test (A) (Anakinra n=11, Etanercept n=16, A+E n=13, Control n=11). Area under the curve (AUC) analysis for panel A is displayed in panel B. $p > 0.05$ between groups by one-way ANOVA.

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Preface to Stem Cell Beta Cell Replacement Therapies

Currently, brain-dead organ donors are the main source of insulin-producing tissue for islet transplant recipients. As for other organ transplant programs, there is certainly a **lack of organ donors compared to the potential recipients**. Finding **alternative sources** would potentially open up islet transplantation to a broader range of diabetic recipients.

Stem cells are a potential limitless source of insulin-producing tissue due to their ability to self-replicate and differentiate. Multiple origins exist for their derivation including human embryonic cells and adult cells. In **chapter 6**, we provide an overview of stem cells, potential sources, progress in the field with respect to insulin-producing cells and potential risks of their use.

Through collaboration with a company in San Diego, we have acquired a source of human embryonic stem cells which have been shown to produce insulin in a glucose-dependent manner after a period of *in vivo* differentiation. In **chapter 7** we propose to replicate these findings and test if shipment of these cells affects their further differentiation and function. We will also determine the potential for teratoma formation in recipients.

Chapter 6

Are Stem Cells a Cure for Diabetes?

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REVIEW

Are stem cells a cure for diabetes?

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ABSTRACT

With the already heightened demand placed on organ donation, stem cell therapy has become a tantalizing idea to provide glucose-responsive insulin-producing cells to Type 1 diabetic patients as an alternative to islet transplantation. Multiple groups have developed varied approaches to create a population of cells with the appropriate characteristics. Both adult and embryonic stem cells have received an enormous amount of attention as possible sources of insulin-producing cells. Although adult stem cells lack the pluripotent nature of their embryonic counterparts, they appear to avoid the ethical debate that has centred around the latter. This may limit the eventual application of embryonic stem cells, which have already shown promise in early mouse models. One must also consider the potential of stem cells to form teratomas, a complication which would prove devastating in an immunologically compromised transplant recipient. The present review looks at the progress to date in both the adult and embryonic stem cells fields as potential treatments for diabetes. We also consider some of the limitations of stem cell therapy and the potential complications that may develop with their use.

INTRODUCTION

Diabetes mellitus is a devastating disease that, according to the WHO (World Health Organization), is expected to affect the lives of 380 million people by the year 2025. It is also estimated that 5% of all deaths in the world are caused by diabetes; a number which will increase by 50% in the next 10 years. TIDM (Type 1 diabetes) is a disease characterized by autoimmune destruction of pancreatic β-cells, whereas T2DM (Type 2 diabetes) is due to systemic insulin resistance and inadequate insulin production by β-cells. In both cases, inadequate glucose control leads to both micro- and macro-vascular complications that account for the morbidity and mortality associated with the disease. Clinical trials have already shown that tight glycaemic control with an intensive insulin regimen can reduce the risk of developing these complications [1,2]. However, even with the use of insulin, most patients with T1DM are unable to maintain their blood glucose levels in the normal range at all times. These patients are also prone to dangerous episodes of hypoglycaemia, a barrier to effective diabetes treatment. These issues have led physicians and researchers to look for other means of controlling glucose levels.

In 1966, the first pancreas transplant was carried out by Kelly and co-workers [3]. Since then, more than 25 000 pancreas transplants have been conducted worldwide, with 1-year graft survival rates in the range of 77% for pancreas transplants alone [4]. However, due to risks relating to the substantial surgical procedure, pancreas transplantation is only suitable for a select subgroup, usually those undergoing simultaneous or previous renal replacement therapy. Therefore alternative approaches are actively needed. This led to the introduction of islet transplantation [5,6]. Now, instead of transplanting the whole pancreas, the insulin-producing islets themselves

Key words: β-cell, pancreatic islet, progenitor cell, stem cell, transplantation, Type 1 diabetes.

Abbreviations: ESC, embryonic stem cell; HSC, haemopoietic stem cell; IE, islet equivalent; IPF-1, insulin promoter factor-1; MSC, mesenchymal stem cell; Ngn3, neurogenin 3; NOD, non-obese diabetic; PDX-1, pancreas and duodenal homeobox-1; T1DM, Type 1 diabetes; T2DM, Type 2 diabetes; UCB, umbilical cord blood.

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Introduction

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procedure, pancreas transplantation is only suitable for a select subgroup, usually those undergoing simultaneous or previous renal replacement therapy. Therefore alternative approaches are actively needed. This led to the introduction of islet transplantation(5, 6). Now, instead of transplanting the whole pancreas, the insulin producing islets themselves could be transplanted, avoiding the need for a major laparotomy. With further refinements, including the Ricordi method for islet extraction(7), the Edmonton Protocol(8) and more recent refinements, islet transplantation became a viable option for the treatment of type I diabetes, but requires potent immunosuppression to facilitate graft survival. Initial results were outstanding with 80% of recipients achieving insulin independence at the one-year point; however, most patients needed to be maintained on a small dose of insulin by the 3-5 years(9, 10). However, more impressive was that these patients achieved better glucose control with avoidance of hypoglycemic events and a substantially improved quality of life(11-13). These results showed that a cure for diabetes is possible through replenishment of the β-cell mass. That being said, islet transplantation has its limitations including a limited source of tissue and the need for chronic immunosuppression. The availability of islet cells from brain dead donors is severely limited; there are only 7,000 such donor per year in the USA(14). The possibility of using living donors has recently been explored in Japan, but does not offer a truly practical approach, as the donor could also be at risk of developing diabetes and has to undergo potentially risky surgery(15, 16). Others have explored the use of alternative animal sources including pig islet xenografts (neonatal porcine islets)(17). Pigs are an ideal islet

source since they are physiologically similar to humans, breed rapidly and produce large litters. In addition, neonatal islets have the potential benefit of proliferation. However, as with other xenografts, there is an enhanced immune response to them; a situation which may be avoided with co-stimulation blockade(18) or encapsulation(19). One must also consider the possibility of introducing infections into the host that were derived from the donating animal species; so called zoonotic diseases. These drawbacks have led researchers to search for other potential sources of glucose responsive insulin-producing tissue including the use of stem cells.

In the following sections we provide a general overview of stem cells, the progress towards their use as a cure for diabetes and some of the limitations encountered thus far.

What are stem cells?

Initially discovered by McCulloch and Till in 1963(20), stem cells are defined by two key features. First, they have the ability to renew themselves through cell division while remaining undifferentiated. Second, when given the appropriate signals, stem cells can differentiate into many specialized cell types. Stem cells can be categorized into two subtypes. Embryonic stem cells are pluripotent cells derived from the inner cell mass of a 4 to 5 day old embryo (blastocyst) and have the potential to form derivatives from all three germ cell layers. Initially derived from mouse embryos(21, 22), it wasn't until 1998 that James Thompson was

able to isolate and continuously culture these cells from a human blastocyst(23). It is important to emphasize here that the human embryonic stem cell is defined as a pluripotent stem cell because it can generate teratomas composed of all lineages when transplanted in immune compromised mice. In the case for mouse ES cells, they are capable of reconstituting an entire mouse following injection of the blastocyst and germline transmission. At the time that human embryonic stem cells were first established in culture, human pluripotent embryonic germ cells were also defined. Since that time, a number of other studies have claimed the generation of pluripotent stem cells derived from the blood(24), the amniotic fluid(25), and the testis(26). In contrast, adult stem cells are found in various adult organ compartments and are more restricted in their ability to differentiate. For example the hematopoietic stem cell gives rise to itself and the progenitor cells responsible for the adult blood lineages. Mesenchymal cells are multipotent stem cells capable of generating for example fat, bone and cartilage. Adult stem cells with restricted organ specific differentiation capabilities also reside within the CNS and skeletal muscle and may exist in all major organs. In these locations they may be functioning as “repair” cells to replenish damaged tissue.

Due to the unique properties of stem cells, a considerable effort has been placed in finding ways to use them to treat various medical conditions especially those conditions where the patient’s endogenous tissues have been damaged. Although as yet there have been no approved treatments using embryonic stem cells, adult stem cell use has become quite common in medical practice. Bone

marrow transplantation employs hematopoietic stem cells (HSC) taken from donor marrow to successfully treat leukemia and other hematologic malignancies. This success has led researchers to explore other uses including the treatment of stroke(27), blindness(28) and even myocardial infarctions(29).

Since type 1 diabetes results from destruction of pancreatic β-cells, the unique regenerative properties of stem cells could be employed to replenish this deficit. Developing a renewable source of islets would circumvent the current supply/demand issues in islet transplantation and provide patients with a long-term source of insulin-producing β-cells. A key challenge will be to prevent autoimmune-mediated destruction of the new cells. For example, hOKT3-ala-ala, an anti-CD3 monoclonal antibody and other immunomodulatory therapies have shown great promise in early clinical trials in mitigating this response(30, 31). Chronic immunosuppression, with its attendant side effects of renal toxicity, increased risk of infection and malignancy, will be required until tolerance, immuno-isolation or HLA-identical cell preparations have been developed. New frontiers in immunomodulation, with calcineurin-inhibitor avoidance and use of costimulatory blockade are moving forward with clinical trials of Belatacept in clinical renal transplantation, and may be one way forward to minimize immunosuppression-related side effects(32, 33).

Stem Cells in diabetes

Although crucial research still continues on improving our current islet transplantation protocol, stem cell investigation has erupted in recent years as a tantalizing treatment for diabetes. Studies have focused on a number of approaches including the use of both pancreatic and non-pancreatic adult stem cells in addition to embryonic stem cells (Figure 6-1). The following sections will provide a review of the progress to date in each of these areas

Pancreatic adult stem cells

The first place that researchers began looking for potential stem cells was in the pancreas itself. Early studies had shown that the pancreas could replenish its β-cell mass when all but 10% of a rat pancreas was removed(34). Although some of this regeneration was due to replication of differentiated β-cells, a significant proportion was thought to be due to cells that were able to dedifferentiate to a more pluripotent form that then produced β-cells. These cells seemed to reside within the pancreatic ducts. Further research using digested human pancreatic tissue proved that this population of ductal cells could, *in vitro,* be cultivated and directed to form islet-like clusters that produced insulin (35, 36). Still further research showed that these ductal cells re-expressed a key transcription factor, insulin promoter factor 1 (*Ipf-1/Pdx-1*), which is known to play a role in pancreatic development and endocrine cell neogenesis (37). Finally, in 2004, Seaberg et al. were the first group to develop a clonal population of adult pancreatic precursor

cells which were isolated from pancreatic ductal cells (38). These cells did express *Ipf-1* as well as several neural precursor markers causing them to develop into neurons and glial cells in addition to pancreatic endocrine and exocrine tissue. In addition, the β-like cells produced both C-peptide and insulin, the release of the latter being positively regulated by glucose. At the same time, other groups were exploring the possibility of stem cells residing within the islets themselves. Zulewski et al. published a series showing that islets, both rodent and human, contain multipotential stem cells which have the ability to differentiate into a number of tissues including pancreatic endocrine and hepatic phenotypes(39, 40). These cells were discovered through their common link to neural stem cells, the filament protein Nestin. Interestingly enough, Nestin-positive cells were also found in pancreatic ducts; providing a potential link between these cells and the previously discovered ductal stem cells(39). Pancreatic stem cells appeared quite promising.

Even with this evidence, there were some that challenged the existence of a pancreatic adult stem cell. Dor et al. reported that using genetic lineage tracing, pre-existing β-cells, rather than stem cells are the major source for new β-cells during adult life in humans and after injury in mice(41). This cast doubt on the studies that had preceded it. Xu et al.(42) recently reported proof that multipotent progenitor cells do exist in the pancreatic ducts of mice and express both IPF-1 and Neurogenin 3 (Ngn3), a transcription factor known to be expressed by endocrine progenitor cells during embryonic development (43, 44). These cells

reappear following pancreatic injury (duct ligation) and give rise to new β-cells. While this conclusively proves that a pancreatic stem cell exists and that β-cells can be formed from non-β-cells, much work still needs to be done to 1) further elucidate the factors that induce certain pancreatic cells to revert to an embryonic mode of development and 2) determine if these stem cells exist in diabetic patients and if they can be somehow activated to promote β-cell formation or isolated and sufficiently expanded ex vivo for transplantation. Extensive studies have been performed in vitro in which adult rodent acinar cells have been transdifferentiated to beta-like cells using combinations of various growth factor and culture methods(45). So far these methods have not proven to be applicable to human acinar cells. Most recently, Zhou et al. have demonstrated that fully differentiated exocrine cells from the pancreas can be directly reprogrammed into cells that closely resemble adult beta-cells, using specific adenoviral delivered transcription factors for IPF-1, Ngn3, and V-maf musculoaponeurotic fibrosarcoma oncogene homolog A (Mafa)(46). Although this does not argue that stem cells exist it suggests that adult pancreatic cells can be re-programmed to become beta-like cells by supplying key endocrine and beta cell “reprogramming” genes.

Non-pancreatic adult stem cells

Hematopoietic progenitor cells

A number of different adult stem cell can be found in the hematopoietic system. These include 1) hematopoietic stem cells (HSCs) which can be isolated form blood, bone marrow or umbilical cord blood (UCB) and 2) mesenchymal stem cells (MSCs) from bone marrow and UCB. The former is responsible for producing all blood cell types while the latter contributes to the formation of mesenchymal tissues, including bone, muscle and fat.

In recent years, bone marrow-derived stem cells have been used successfully to treat a number of hematologic malignancies due to their ability to reconstitute the hematopoietic system. Recent research has also shown them to have the ability to transdifferentiate into a number of other cell lineages including liver, brain, lung and even gastrointestinal tract cells (47-50). In addition, UCB represents an abundant and easily accessible resource showing a heightened ability to differentiate when compared to bone marrow or peripheral blood. For these reasons, a significant amount of work has focused on the possibility of using hematopoietic progenitors to replenish the β-cell population in type I diabetes.

In terms of bone marrow cells, an early mouse study proved promising, showing that these cells had the ability to differentiate *in vivo* into functionally competent

β-cells (51). The group injected bone marrow from male donor mice, whose cells were genetically modified to produce a fluorescent protein if insulin was produced, into irradiated female donor mice. Within pancreatic islets in the recipient mice were found fluorescent protein-producing cells that contained a Y chromosome. Thus, they were able to show that bone marrow cells were able to populate the pancreatic islet cells. In addition, this treatment was specific, extra-pancreatic cells did not express the same fluorescent protein. Similar experiments were carried out by other groups using chemically induced diabetic mice but without similar results(52-54). They were able to show that bone marrow cells can be targeted to the pancreas and that hyperglycemia can be reversed(52); however, they failed to provide evidence of differentiation of bone marrow cells into β-cells. These researchers felt that the marrow cells facilitated endogenous pancreatic regeneration possibly via conversion to endothelial cells or generalized anti-inflammatory activities but they themselves did not become functional beta cells.

At the same time, other groups were looking at blood stem cell populations in the NOD mouse model (55-58). Widely used in diabetes research, the NOD mouse is an autoimmune diabetes model. Once again an initial report looked promising. Here, NOD mice were given a syngeneic islet renal subcapsular transplant to treat their hyperglycemia. Mice were then given donor splenocytes and complete Freund's adjuvant (previously shown to prevent autoimmune diabetes in NOD mice(59)) and after 40 days the islet graft was removed(55). Normoglycemia

continued. They hypothesized that the donor splenocytes differentiated into insulin-producing cells to regenerate the new β-cell mass. Three further studies partially replicated these experiments showing that a proportion of mice could regain endogenous β-cell function but could not find any evidence that the splenocytes themselves replenished the β-cell mass (56-58).

Similar experiments were performed using UCB cells. In one study, T-cell depleted mononuclear cells were transplanted into newborn mice(60). As the mice developed, 0.65% of the insulin-positive cells in the pancreas were shown to be of UCB origin; a process that was both dependent and independent on fusion mechanisms. Another group subjected UCB cells to previously reported embryonic stem cell culture conditions, leading to production of C-peptide(61, 62).

Taking these cells one-step further, a cohort of type 1 diabetic children was transplanted with autologous UCB cells(63). Here, they showed lower average daily insulin requirements and lower average HbA1c levels. Unfortunately, it is still unclear whether this was due to differentiation of the transplanted cells or altered immune regulation. Still even more promising are the recent studies showing improvement in type 1(64) and type 2 diabetes(65) when autologous stem cells were transplanted. Although the patients in the former trial developed a number of complications, these trials provide promising results for the use of autologous hematopoietic stem cells in the treatment of diabetes. In addition, the

use of a patient's own cells would likely avoid the ethical dilemma associated with their embryonic counterparts.

Other non-pancreatic adult stem cells

Since the pancreas is embryologically derived from endodermal tissue, a logical place to search for pancreatic stem cells is in other endodermal-derived organs. Both the liver and the small intestine share a common lineage origin with the pancreas and as such, both have been extensively studied as potential sources of pancreatic β-cells.

Early work on the liver uncovered multipotent cells able to differentiate into both hepatocytes and bile duct epithelium. These were termed "hepatic oval cells" (66). Using this knowledge, researchers began the search for ways to induce the differentiation of these liver cells into insulin-producing cells. Once again, PDX-1/IPF-1 proved a useful tool with multiple groups successfully transdifferentiating rodent hepatic cells into insulin-producing cells via multiple genetic approaches (67-76). Most groups used adenovirus-mediated gene transfer techniques to introduce transcription factors (especially PDX-1) into hepatic cells *in vivo* (67, 69, 73, 74, 76, 77) while others found that in vitro high glucose concentrations and/or transplantation into a diabetic mouse were necessary to stimulate hepatic cell changes (68, 72). Regardless of the method used, amelioration of hyperglycemia was achieved in these mouse models; giving hope to researchers searching for extra-pancreatic sources of insulin.

Other potential stem cell sources have also been studied including the small intestine(77-79), salivary glands (80) and adipose tissue (81). Varying success has been achieved in this regard and if a non-pancreatic stem cell source of β-cells is to be utilized in a cure for diabetes, it seems that the liver could be a likely source based on its close embryological proximity and the results obtained to date. Although issues remain, including the expandability and durability of these cell lines, the ability to achieve fully differentiated β-cell phenotypes and the potential necessity of islet structure formation, hepatic production of insulin has the potential to become a viable source for β-cell replacement in the years to come.

Human embryonic stem cells

Embryonic stem cells (ESC) have received a heightened amount of interest in the last decade owing to their pluripotency and their ability to self-renew. These unique characteristics have led many researchers to explore their use in a number of medical conditions, especially those with a degenerative or destructive etiology. Type I diabetes fits in this realm of diseases since the initiating insult is an autoimmune destruction. The theory has developed that if embryonic stem cells could be directed to differentiate into pancreatic islet cells, and these cells could then be implanted in patients with diabetes, then the β-cell deficit would be overcome. This differentiation task, however, has proven to be complex and difficult.

The first attempts at producing islet cells *in vitro* from mouse embryonic stem cells was published in 2000(82). Here, the group led by Soria developed insulin-secreting clones from a genetically engineered and drug-selected mouse ESC line. They were able to transplant these cells into diabetic mice and were able to achieve a degree of amelioration of hyperglycemia that lasted for a few months(82). Unfortunately, the rate of insulin-positive cell production was quite low, likely because their cell selection process was conducted before full differentiation. As such some of the cells in their final group were non-islet insulin-producing cells (eg. neural cells). Over the next few years, a number of other groups published results with varying degrees of success. These initial studies employed both mouse(61, 83-88) and human ESCs(89, 90) and were limited by final cell homogeneity(61), immaturity of the differentiated cells(90), low numbers of insulin-producing cells(91) and a poor insulin-response when the cells were exposed to glucose(87, 89). In general, all of these studies lacked thorough analysis and demonstration of the sequential developmental steps required to ensure formation of foregut pancreatic cells capable of endocrine hormone production. Although these early reports brought some hope, it was certainly proving difficult to create reliable insulin-producing cells with β-cell phenotype from embryonic stem cells.

To add to these difficulties, other groups were beginning to question whether these cells were actually producing insulin at all. Three separate teams were able to show that the majority of the claimed differentiated pancreatic “insulin-producing” cells did not, in fact, produce any insulin(92-94). They argued that the insulin found in these cells was due to its uptake from the surrounding media. In support of this they showed a lack of C-peptide production(92-94) and a lack of intracellular insulin once the cells were cultured in insulin-free media(93). For further progress to be made with ESC’s, proof of C-peptide production would have to be shown.

This setback forced researchers to rethink their differentiation strategies. The stages and gene expression involved during normal pancreas development were already known(95-97), including the first major differentiation stage in the development of the endocrine pancreas, the definitive endoderm germ layer(95). Kubo and colleagues were able to define the exact culture conditions necessary to convert mouse ESCs into definitive endoderm(98). This paved the way for D’amour et al. to refine this protocol and produce a near 100% pure definitive endoderm cell population(99). The same group extended their work on definitive endoderm and developed a five stage *in vitro* differentiation process modeled after *in vivo* development of the pancreas(100). In this work they demonstrated production of pancreatic endocrine-hormone producing cells that contained both insulin and C-peptide. In fact, the insulin content of these cells was in the range of that for mature human islets. Unfortunately, although C-peptide was released

from these cells in response to various stimuli, including KCl and cAMP, neither c-peptide nor insulin was released in response to glucose. Hyperglycemic responsiveness is a crucial characteristic that is needed for any potential cellular diabetic therapy. In order to overcome this deficit the group once again looked at earlier research in which fetal human pancreatic anlagen was successfully isolated and grafted into rodents resulting in functional human islet formation. This time they stopped their in vitro differentiation at a point at where the cells resembled a 6-9 week old embryo; they were committed to the pancreatic lineage but had not yet transitioned to the endocrine progenitor state (101). A prior report had demonstrated that 6-9 wk human embryonic pancreas (primarily pancreatic epithelium as opposed to endocrine cells) grafted into an immunodeficient, diabetic mouse was able to further develop and reverse hyperglycemia(102). Using this knowledge, the group transplanted their differentiated cells into the epididymal fat pad of immunodeficient mice. Although C-peptide levels were low one month post-transplant, at the 90 day-point levels were approaching those seen with transplantation of 3000-5000 human islets. Insulin release was also measured, and was shown to be released in a glucose-dependent manner. This allowed the cells to both recover mice from STZ-induced diabetes as well as prevent it.

These recent discoveries have paved the way for embryonic stem cells to become a strong candidate for cellular replacement therapy in Type 1 diabetes. However, we are still likely a number of years away from any potential clinical

trials with a number of questions still left to be answered including the long-term stability of stem cell derivatives and their potential side effects.

How far away is cellular replacement therapy for diabetes?

While researchers have made enormous strides toward using stem cells as a potential treatment for type I diabetes there are a number of issues that need to be addressed. These include concerns of both a scientific and societal nature (Table 6-1).

Firstly, before any new treatment is used in the human population, it of course needs to undergo rigorous testing and screening for potential side effects. This concern for safety is likely even more heightened when it comes to stem cells. One complication that has already arisen in the mouse models is the formation of teratomas with the potential for malignancy. This is especially a concern with embryonic stem cells where groups have already observed teratoma formation when grafts were histologically assessed(86, 88, 101). These tumors form due to the implantation of undifferentiated cell populations into an immunodeficient host; such as would be the case if these cells were introduced into a patient on necessary immunosuppressives. It would be difficult to treat patients with a cell replacement product for diabetes if it could not be demonstrated to be safe with respect to teratoma formation. Future protocols will therefore need some form of purification or screening step in order to eliminate and screen for the presence of unsafe cells respectively. Of course if the replacement therapy could be

administered without immunosuppressive drugs, the possibility of teratoma formation would no doubt be lessened.

The pancreas is a very complex organ with many functions both endocrine and exocrine in nature. Endogenous β-cells develop through a regulated pathway to eventually become the insulin producing cells which regulate euglycemia. The mature cells are part of an integrated milieu of cells and cellular signals together with their cellular products. Even the islets transplanted in current clinical islet transplant programs contain β along with α and δ-cells. In addition, endogenous islets are situated in a complex array of vascular and neural supports. How will stem cell derived products behave once transplanted? Depending on the transplant site, will they be able to develop these same vascular and neural connections? Even though some groups have shown production of glucagon and somatostatin in their cell populations, how will these cells interact once transplanted into an unfamiliar environment? Will it be necessary to have a complete islet structure with the appropriate endocrine hormone composition or will it be sufficient to have appropriate numbers of β-cells. Recent work has demonstrated that purified β-cell preparations are sufficient to treat the diabetic condition in rodents(103). In addition, it is likely that β-cells are able to adapt to changes in their glucose environment and adapt to insulin resistance through both neogenesis and cell replication(104). Will stem cell-derived β-cells have these abilities or even worse, will increased insulin resistance cause these β-cells

to expand uncontrollably? Further studies will no doubt need to address these issues.

Although a cellular-based replacement therapy for diabetes would overcome one of the major limitations of our current islet transplantation protocol, it is still likely to be subject to the other major limitation. Unless a protocol is developed where stem cells are derived from a patient's own cellular population (and even here the issue of the autoimmune insult which caused the disease needs to be addressed), some form of immunosuppression or an immunoisolation delivery strategy will be required. Clearly our knowledge of the immune system and therapies targeted at diminishing its effects have made great strides, but patients are faced with unpleasant and at times unbearable side effects from immunosuppressive agents. So although stem cells could conceivably circumvent the need to rely on organ donation for a source of insulin-producing tissue, they may do nothing to relieve the toxicity associated with the post-implantation drug therapy – unless additional immunomodulatory regulatory stem cells are co-transplanted or specific tolerization strategies are developed. Any future stem cell-related therapies will no doubt be facilitated by improvements in the tolerance of our current anti-immune therapies. Underlying this point is the need to continue with the current research into islet transplantation since any further advances made there will no doubt positively impact the development of any cellular-based diabetes therapy.

When considering current clinical islet transplantation programs another interesting issue arises. Current guidelines employ a minimal islet implant mass of 10,000 IE/kg, usually obtained by harvesting two pancreases(9). Even with this amount of islets, most patients need to return to a small amount of insulin at the two to three year mark. It still remains to be seen how stem cells can compare, in terms of insulin production and potency, to this amount of islets. Although the group led by Kroon (101) have stated that their stem cell-derived products are achieving a production rate of C-peptide equivalent to 3,000-5,000 human islets in their mouse model, this is far short of the levels needed to support an adult human. The scale-up potential of stem cells will therefore need to be further studied to provide an excess of transplanted cellular reserve.

The last issue, and certainly by no means the least important, is the intense ethical debate that forms from any discussion of stem cells. Beginning with the cloning of Dolly the sheep in 1997, cultural fires have ignited with any mention of cloning or genetic engineering. These fires have now spread to the field of stem cell research. Here, it seems the issues revolve mainly around embryonic stem cells and their derivation. In short, embryonic stem cells are usually derived from unused embryos at in vitro fertilization clinics. Full informed consent needs to be given by the donor before these cells can be used. Unfortunately, the embryo, in most cases, needs to be destroyed to harvest the cells it contains. It seems that the majority of the controversy develops from this derivation process and the question of when life actually begins. On the one hand are those that believe that

stem cell research violates the sanctity of life. They are of the mind that life is inviolable and begins when a sperm fertilizes an egg. They are in direct contrast to those that take a more utilitarian view on the issue where the potential benefits, in terms of cellular therapies for medical conditions, outweigh the potential costs. While this debate continues, the full extent of its impact on research using stem cells remains to be seen. Although adult stem cells will likely avoid much of the negative publicity generated by their embryonic cousins, any potential clinical uses involving stem cells will need to be accompanied by a thorough explanation of their derivation. Although unlikely to end the debate, it will hopefully ease some of the tension that has built up around this topic.

A longer term solution to the hESC ethical dilemma will likely be the Induced Pluripotent Stem Cell approach (105, 106). In this case adult cells are reprogrammed to the pluripotent state to be subsequently differentiated to functional β-cells. The further safety concerns associated with ex-vivo gene therapy with oncogenes which together with a better understanding of the genetic and epigenetic stability adds a further safety burden likely to be solved in the future.

Conclusion

Are stem cells a “cure for diabetes”? While great strides have been made towards using stem cells as a cellular replacement therapy for type 1 diabetes, many issues still remain. It would seem that embryonic stem cells have the advantage thus far owing to their enhanced potential for directed differentiation and replication. Unfortunately, it is embryonic stem cells that also are fraught with potential complications including a tendency to form teratomas and ethical debate revolving around their derivation. That said, in the last ten years, we have been able to use a knowledge of normal embryologic development to differentiate stem cells into insulin-producing tissue with the ability to reverse diabetes in an animal model. Who knows what the next ten years will bring.

Figure 6-1. Potential Stem Cell Sources for Insulin-Producing Cells

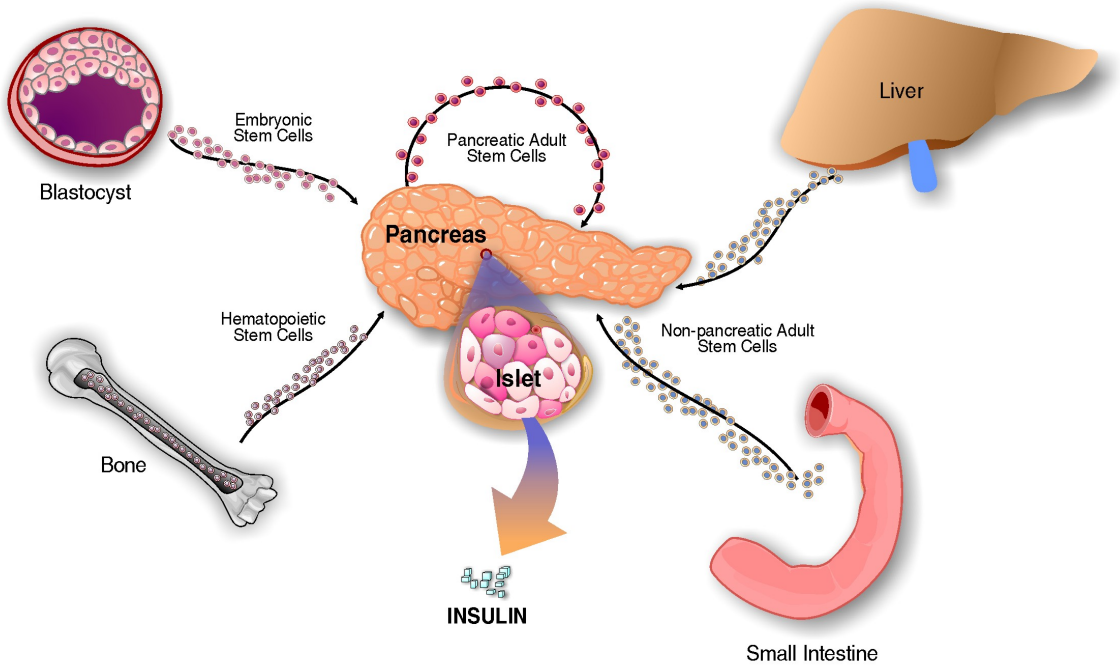

Table 6-1 Issues which must be resolved before a clinical application of stem cell-derived products can be attempted

Issues related to the use of stem cells in the treatment of type I diabetes
• Potential risks, including teratomas • Challenges related to the transplantation of isolated cells lacking vascular and neural support • Need for immunosuppression • Ethical acceptance • Engraftment of a non-marginal mass of insulin-producing cells for human use (exceeding 15,000 IE/kg human islet equivalent function)

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Chapter 7

Human Embryonic Stem Cell-Derived Pancreatic Endoderm Cells Reverse Diabetes after Transplantation in Immunodeficient Mice after Shipment, but form Teratomas at Late Time-Points

A version of this chapter has been submitted to *Diabetes* for peer review and publication

Introduction

Diabetes is a significant worldwide problem with an estimated prevalence of 366 million by the year 2030 (World Health Organization) (1). A 10% sub-set of patients have autoimmune type 1 diabetes with destruction of islet beta cells. While insulin is the cornerstone of treatment, in highly selected cases, transplantation of human islets and immunosuppression is being increasingly applied to stabilize glycemic control, and to potentially reduce risk of complications. If this approach is to be more widely applied, a source of insulin secreting beta cells that does not depend on a limited human cadaveric donor source, will be key to more broad application, with options including living donor islet transplantation (2, 3), xenotransplantation (4-6) and human insulin secreting embryonic or adult pluripotential-derived stem cells.

Human embryonic stem cells, derived from the inner cell mass of a 4 to 5 day old embryo have the ability to self renew in addition to developing into all three germ layers. While this could potentially provide a limitless source of insulin-producing tissue, their production has proven challenging. Early attempts suffered from low numbers of insulin-positive cells (7), cell immaturity (8) and a poor insulin-response when stimulated by glucose (9, 10). In 2005, a human embryonic derived cell line called Cyt49 was developed, with high purity pancreatic endoderm cell (PE) population (11). Further differentiation provided cells which matured within immunodeficient mice and responded to hyperglycemia with the production of human c-peptide and human insulin (12). Furthermore, these cells

were more resistant to streptozotocin, and were able to reverse the diabetic state in these immunodeficient mice. While promising, Matveyenko et al. questioned the potency of these Cyt49 derived cells, as they failed to demonstrate an insulin or c-peptide response to glucose challenge(13). Further proof of *in vivo* functionality of these cells is therefore needed before considering these cells for potential clinical application. The concept of moving forward a limitless stem cell derived insulin secreting source to the clinic would require generation and transfer of cells to clinical sites. We therefore set out to evaluate the potential of inter-center and between country transportation of the Cyt49 cell line, and to more robustly characterize the insulin secretional capacity of these cells *in vivo*.

One major concern of several human embryonic stem cell derived insulin secreting cells is their potential to form teratomas. While teratomas are generally benign, and with low risk for malignant transformation, the capacity for local, uncontrolled expansion creates a substantial barrier to potential clinical application, an issue that has not been studied in detail in prior studies (12, 13). We therefore set out to investigate the short-term metabolic function of Cyt49 cells after shipment from manufacturing to a remote facility, and to further study the long term risk of teratoma transformation after transplantation into immunodeficient mice.

Materials and Methods

Animals and Reagents

Immunodeficient B6-RAG $^{-/-}$ mice (B6.129S7-*Rag1tm1Mom*/J) were obtained from the Jackson Laboratories (Bar Harbor, ME) and housed under specific pathogen-free conditions. All animals were cared for according to the guidelines of the Canadian Council on Animal Care, and ethical approval was obtained from the animal welfare committee at the University of Alberta. All reagents were obtained from Sigma Aldrich (Oakville, Ontario, Canada) unless otherwise specified. Approval to use human embryonic stem cell-derived tissue was obtained from the Canadian Stem Cell Oversight Committee (SCOC).

Shipment of Cyt49 derived human embryonic stem cells

Cyt49 cells were obtained as a generous gift, without cost, and under a material transfer agreement from Viacyte, San Diego, CA, USA. Cells were shipped in a 50 ml conical tube in media, and transferred by commercial airline and truck carrier (Federal Express Inc). An electronic thermometer was enclosed with each shipment showing stability of temperature en route. Cells arrived within twenty four hours after shipment, and were transplanted into mice on the day of arrival. N=20 mice were implanted with human embryonic stem cell derived PE cells over 3 separate shipments, and were followed for one year post transplant.

Cyt49 Cell Transplantation

Human Cyt49 derived cells were implanted in the renal subcapsular space in these immunodeficient mice, following standard protocols used previously in islet transplantation (14). In brief, mice were placed under anesthesia using 1% isoflurane. A left flank incision was made and the kidney exposed. Twenty microlitres (20uL) of Cyt49-derived cells ($2 \times 10^6 – 2 \times 10^7$) were gently spin pelleted and placed under the kidney capsule using PE-50 tubing, and the capsule cauterized to seal the site and prevent cell spillage. Animals were allowed to fully recover from anesthesia. Mice had constant access to food and water and gained weight throughout the study.

Glucose tolerance/C-peptide release

At two, four and five months following implantation, mice were fasted overnight before being given a 3 g/kg intraperitoneal dose of 50% Dextrose. Blood was obtained via tail vein at baseline, 60 minutes after injection. Blood was allowed to sit at room temperature for 30 minutes in microfuge tubes before being spun at 10,000 rpm for 30 minutes. Serum was collected and frozen at -20 °C until mass analysis could occur for human c-peptide levels using an ELISA kit (ALPCO diagnostics, Windham, NE).

Assessment of diabetes prevention

Four months after Cyt49 cell implantation, transplanted B6-RAG$^{-/-}$ mice (n=10) and naïve control mice matched for age and weight (n=10) underwent serial low-dose streptozotocin (STZ) treatment (60mg/kg i.p. on 3 consecutive days). The purpose of the multiple low-dose STZ treatment was to capitalize on the differential sensitivity of the native beta cells compared to the more resistant human Cyt49-derived beta cells to STZ toxicity, and thereby to provide evidence of mature insulin reserve within the transplanted cells (12). Blood glucose was monitored daily via the tail vein. At ten days post STZ, the Cyt49 graft-bearing kidney was removed and blood glucose monitored for a further 48 hours to confirm hyperglycemia, and to establish that the transplanted cells were the source of human insulin.

Histological analysis

Pre-implant Cyt49-derived cells (post-shipment) were fixed in formalin for histological analysis. Graft-bearing kidneys were removed, sectioned and placed in formalin. After paraffin embedding, slides were cut and stained with hematoxylin and eosin (H&E). Further unstained slides underwent immunofluorescence staining for insulin, glucagon, somatostatin and pancreatic polypeptide.

Results

Cyt49-derived cells implanted under the kidney capsule of immunodeficient mice mature over time, and are glucose responsive

Cyt49-derived cells were implanted under the kidney capsule of immunodeficient B6-Rag$^{-/-}$ mice and allowed to further develop and mature. Over the first twenty weeks after implantation there was a steady decline in the fasting blood glucose with a significant reduction as compared to naïve mice of a similar age and weight (Figure 7-1). The mean fasting plasma glucose in the Cyt-49 transplanted mice was significantly lower than the naïve control untreated mice (mean 5.7 ±0.2 vs 3.2 ±0.1 mmol/L, $p<0.05$), possibly reflecting different glycemic set-points for human vs mouse islets (15). By comparison, the mean fasting blood glucose of B6-Rag$^{-/-}$ mice one month after transplantation with an engrafted human islet graft (1500 islet equivalents) is 3.3 ± 0.2 mmol/L. There was no evidence of progressive or symptomatic hypoglycemia over time, and after maturation, glycemic levels remained stable. At eight, 12 and twenty weeks after implantation, mice were fasted overnight and underwent a glucose tolerance test with blood sampling at baseline and sixty minutes after dextrose bolus. At all time points, there was a significant increase in serum human c-peptide >2.3 times baseline values (Figure 7-2A). In addition, these mice displayed increasing baseline human c-peptide levels over time ($p<0.05$ by two-way ANOVA). A portion of Cyt49 cells received were maintained at room temperature to simulate a further 24 hour delay in shipping before being implanted. This did not affect the

ability of the PE cells to develop and function as these mice displayed significant increases in human c-peptide over baseline upon administration of dextrose (Figure 7-2B). Serum from naïve B6-Rag$^{-/-}$ mice was analyzed with each ELISA as a negative control; in all cases there was no human c-peptide detected (not shown), confirming the specificity of this assay in our hands.

Cyt49 cells are able to prevent streptozotocin-induced diabetes

Six months after implantation, Cyt49 and naïve mice (of similar weight and age) underwent serial STZ treatment as described above. The naïve mice all became acutely diabetic, while the Cyt49 transplant recipients remained normoglycemic (Range 3.4 - 6.9 mmol/L, Figure 8-3). At day 10 post STZ, the graft-bearing kidneys were removed and blood glucose followed for a further 48 hours. All Cyt-49 treated mice promptly became hyperglycemic, displaying blood glucose readings over 16.5 mmol/L (mean 20.2 ± 1.1 mmol/L).

Cyt49 cells mature *in vivo* displaying mature pancreatic endocrine phenotype, but with eventual formation of teratomas by one year

At baseline (shipped cells), 8 weeks and one year after implantation, mice were sacrificed and their graft-bearing kidneys removed for histological analysis. Figure 7-4 (top panels) displays H&E staining of shipped cells, and grafts 8 weeks and one year post-implantation. Eight-week grafts displayed duct-like structures surrounded by a stromal matrix. To assess the development of

pancreatic endocrine cells, graft sections were evaluated by immunohistochemistry (Figure 7-4, middle and lower panels). While there was early evidence of insulin in shipped PE cells, this increased markedly in Cyt49-derived grafts by eight weeks and at one year. Somatostatin, glucagon and pancreatic polypeptide co-localization with insulin was evident by eight weeks and became more apparent by one year, demonstrating all of the typical functional components of a mature human islet.

We followed a cohort of 12 mice for one year following Cyt49 cell transplantation. All 12 mice survived, and there was no external evidence of a mass or other concerns. Mice were then sacrificed, and Cyt49-graft-bearing kidneys removed for histological analysis. Macroscopically, we found evidence of cystic expansion of the graft site in all 12 cases (100%), with the transplant site expanded to several times the size of the native kidney (Figure 7-5A). Of note, this finding occurred with all three different shipments of Cyt-49 cells. The microscopic analysis confirmed the presence of ectoderm, mesoderm and endoderm, with dominant cystic mucinous content, consistent in all cases with formation of benign teratoma. There was no direct invasion of the renal parenchyma, and no evidence of extra-renal capsular invasion or metastasis. These were ‘asymptomatic’ in all cases, and did not appear to interfere with activity or feeding, and the mice appeared well. Without termination, we would not have been aware of this finding.

Discussion

The goal of this study was to examine the potential of a human embryonic derived insulin-secreting cell line (Cyt49, Viacyte Inc) to function *in* vivo after shipment to a remote center. Shipment of a potentially renewable source of surrogate beta cells will eventually require validation, after cells are initially prepared at a central production facility, and then distributed to recipient centers. This model already exists in a number of clinical islet transplant centers, where isolated islets are prepared and transported for transplantation at remote sites, (16, 17). Herein, we clearly demonstrate the capacity of the Cyt49-derived cells to fully correct the diabetic state after transplantation and maturation in immunodeficient mice, confirming the previous work of Kroon *et* al. (12). We confirmed that cells remain completely viable despite a period of up to 24 hours in shipment, and under temperatures up to 23°C. We confirmed that the Cyt49-derived cells secrete human C-peptide and human insulin in response to stimulation *in* vivo, and using the multiple low-dose STZ model previously described by Kroon *et al.* we confirm that these Cyt49 stem cells can maintain normoglycemia after maturation, and that graft explantation led to acute hyperglycemia in all cases.

Once implanted under the kidney capsule, cells underwent a maturation and development period with release of human c-peptide clearly detectable by 8 weeks post implantation. Moreover, this release was sensitive to glucose with significant increase observed in response to a dextrose bolus. This is in direct

contrast to a recent study using similar Cyt49-derived cells(13) but confirms the original results of Kroon *et al*. (12).

We observed relative hypoglycemia with Cyt49-treated mice maintaining stable glucose levels of a mean 3.2 mmol/L (range 3.1 – 3.6 mmol/L) by 20 weeks post transplant, but levels remained constant thereafter, and were no different at one year than at 20 weeks. Any potential concern of progressive, uncontrolled and unregulated hypoglycemia as Cyt49 stem cells mature and expand does not appear to be the case, and it is reassuring to note that islets isolated from our clinical islet transplant program and transplanted into similar immundeficient mice typically achieve almost identical levels of relative hypoglycemia, suggesting that the glycemic set-point of both primary human islets and Cyt49-derived mature stem cells is similar, and significantly lower than the natural set-point in mice.

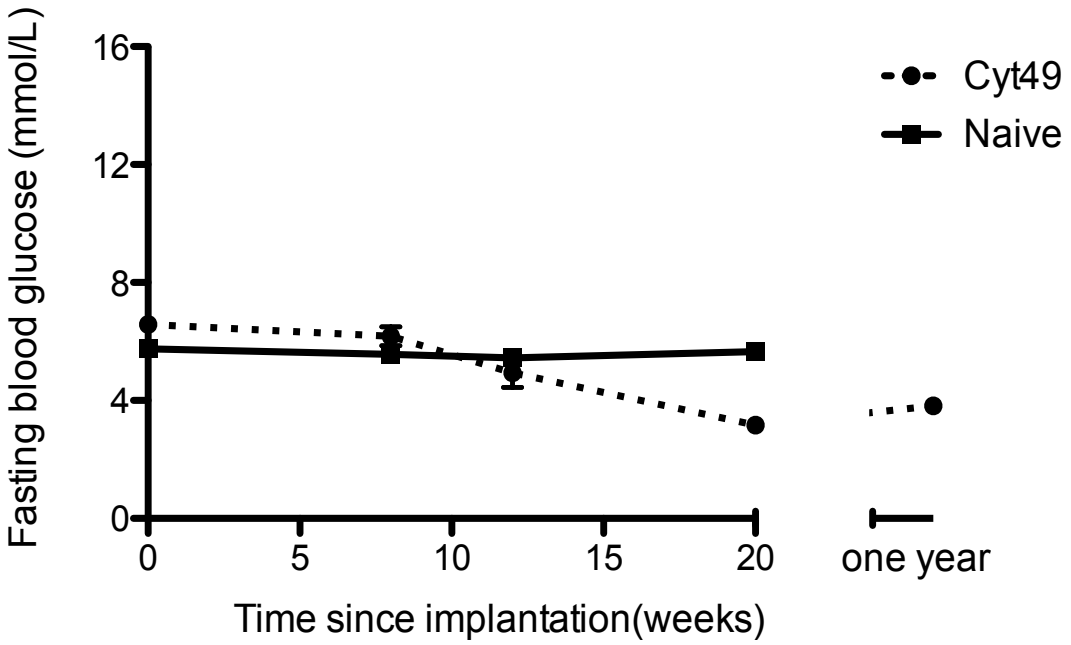
The observed human C-peptide response in the current study was lower than those reported previously with the Cyt49-derived cell line (12). Differences between the two studies include: a) shipment between centers, b) use of $Rag^{-/-}$ mice instead of SCID-Beige mice, c) use of the renal subcapsular space for cell implantation rather than the epididymal fat pad, but similar cell mass was transplanted in both cases (18). Nonetheless, the Cyt49 cell performed with full metabolic reserve after shipment, and clearly controlled glycemia until explantation.

The observation that all 12 mice followed out to beyond one year developed expanded cystic masses consistent with teratoma remains of concern, especially for a cell line that has potential for clinical application. Kroon *et al*. previously reported rates of 2.2% teratoma risk, but of note, with a further 6/46 (13%) showed monodermal forms of teratoma, but their transplants were followed out for shorter duration (12). We found no evidence of malignant transformation or local invasion, although the mucinous cystic nature of these masses represents a mass of approximately 5-fold the size of the native adjacent kidney. Teratoma formation is a recognized risk of human embryonic stem cell derived cells. We only observed the teratoma masses at the time of termination, and therefore cannot comment on the temporal development, or whether they would continue to expand over time, or potentially interfere with beta cell function. The absence of an immune surveillance system in the $Rag^{-/-}$ mice has been established as favoring an environment that supports unregulated growth, and it therefore remains unknown whether a similar risk would pertain in the clinical environment (19, 20). The fact that patients would require systemic immunosuppression to support the survival of these allogeneic cells, could be a potential confounding element. The drug sirolimus has been shown to have anti-angiogenic and anti-tumoral effects, and this agent could potentially mitigate teratoma formation, but this requires more formal study (21-24). Other human embryonic stem cell derived lines (hESC) transplanted into the renal subcapsular site have also demonstrated high rates of teratoma formation dominated by cystic elements (20). Our results confirm these previously well described risks. If Cyt49-derived

hESC's or other hESC-derived lines are to move forward to the clinic, it is evident that effective means to prevent and control teratoma must be sought. High-throughput screening by flow-cytometric sorting, and more homogenous or pre-matured endodermal lines, might reduce this risk. The use of specific cytolytic monoclonal antibodies could also be used to selectively deplete non-islet elements with a cell line. Furthermore, transplantation of stem cell derived beta cells into a more accessible and resectable site rather than the intraportal liver site, will likely be mandatory if risk of transformation is not guaranteed. Therefore, contained, subcutaneous pouch devices are being developed with the concept of graft containment in mind (13, 25-27).

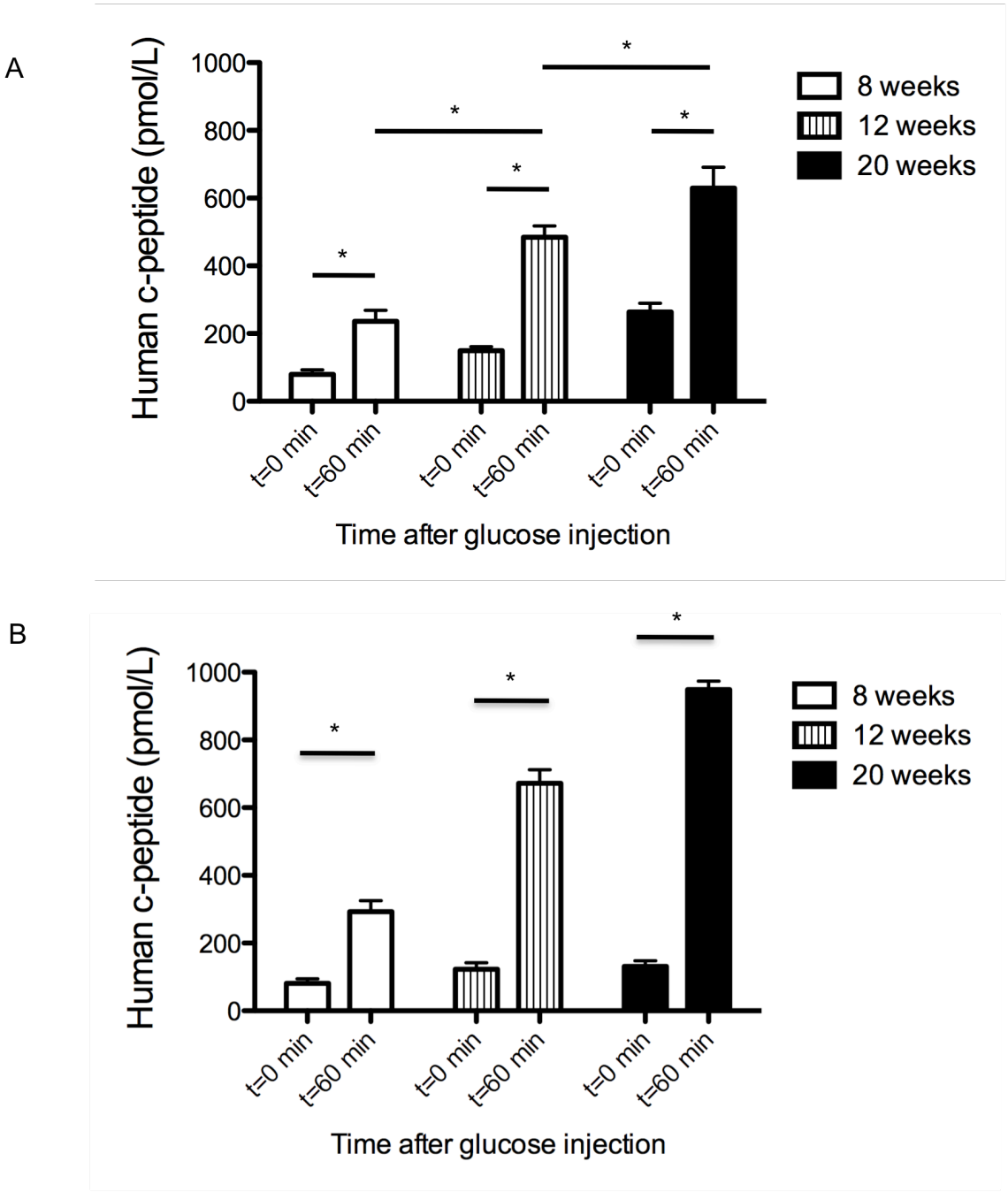
While islet transplantation has seen promise as a treatment for diabetes, finding a limitless source of insulin-producing tissue would be a major advance for both Type 1 and potentially Type 2 diabetes. Human embryonic stem cell-derived pancreatic endoderm cells could potentially fill this need. However, the risk of teratoma formation needs to be addressed. Altering the transplant site or implanting the cells into an encapsulation device may prevent development of these tumors and should be studied further.

Figure 7-1 Mice implanted with PE cells show reduced fasting blood glucose which stabilizes over time



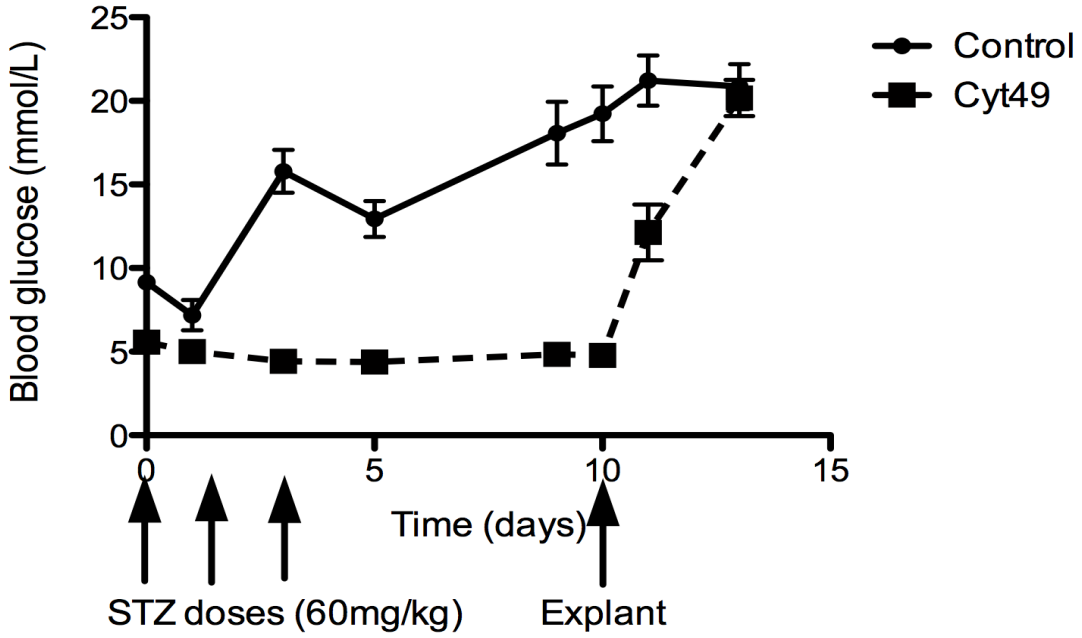
Immunodeficient mice were implanted with cells derived from a human embryonic stem cell line (Cyt49) . At eight, twelve, twenty and 54 weeks after implantation, mice were fasted overnight and blood glucose was taken. Blood glucose was also sampled from naïve mice of similar age and weight as a control. (Mean blood glucose at 20 weeks: naïve 5.7 ±0.2 vs Cyt49 3.2 ±0.1 mmol/L, $p<0.05$)

Figure 7-2 PE cells display endocrine response to glucose in vivo



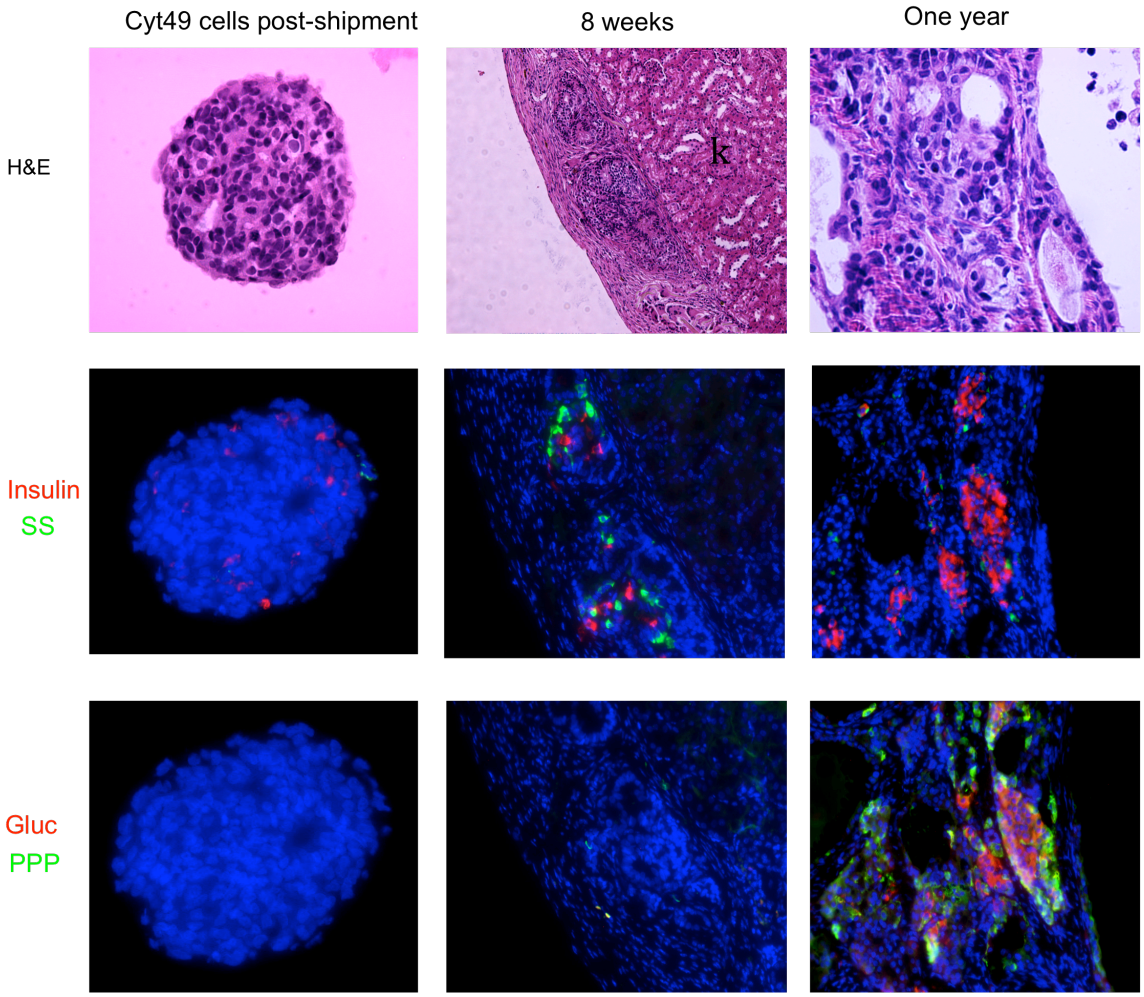
Eight, twelve and twenty weeks after implantation with Cyt49-derived cells, mice were fasted and underwent glucose tolerance testing. Serum was collected at baseline and sixty minutes after intraperitoneal injection of dextrose. Samples were analyzed on a human c-peptide ELISA kit using naïve B6-Rag$^{-/-}$ serum as control (not shown). (A) Cells received 24 hours shipping and (B) cells maintained at room temperature for a further 24 hours to simulate a 48hr shipment (*= $p < 0.05$)

Figure 7-3 Cyt49-derived cells overcome streptozotocin-induced diabetes



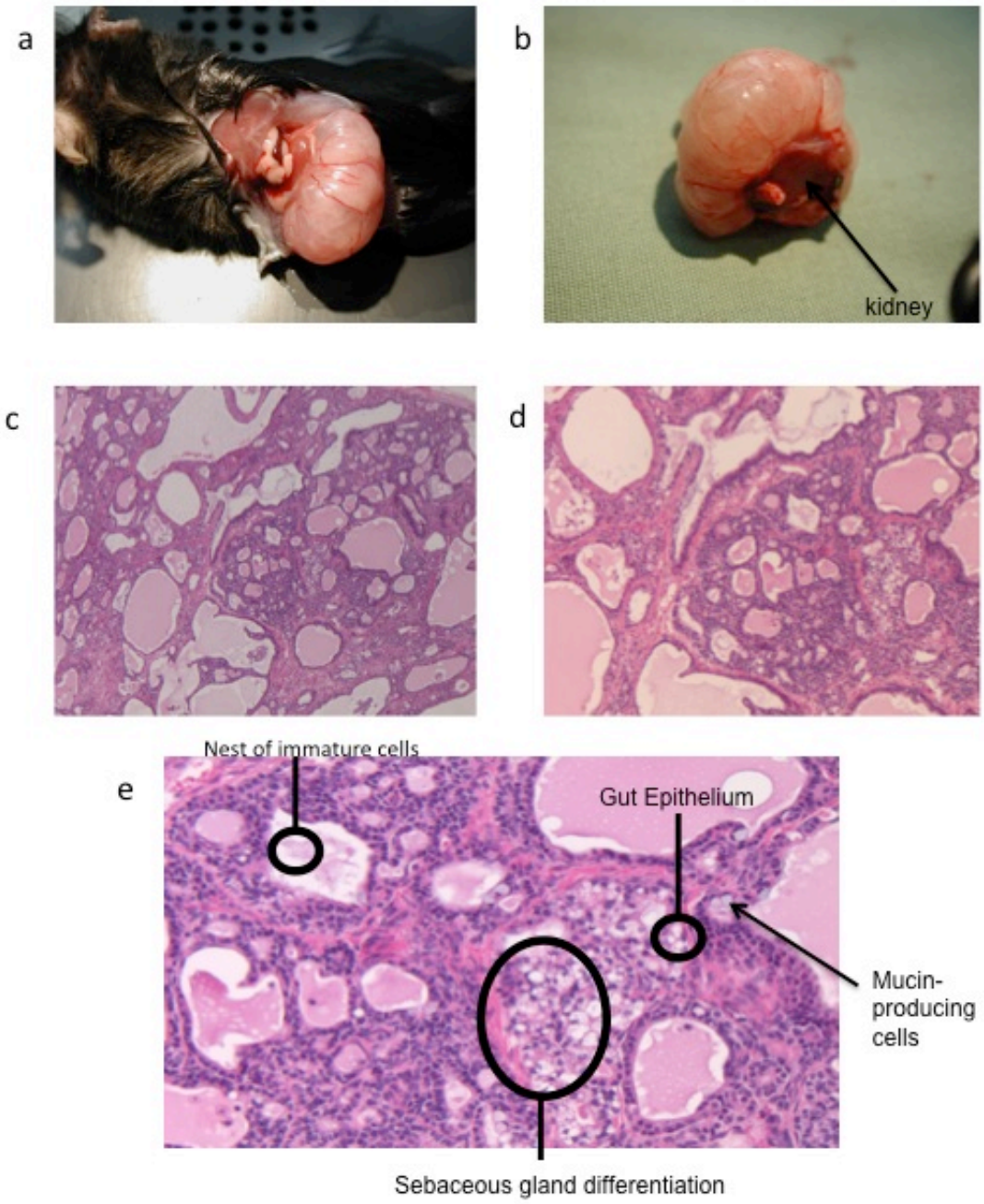
Six months after implantation, N=10 B6 Rag$^{-/-}$ with kidney subcapsular Cyt49-derived cell grafts and N=10 naïve B6 Rag$^{-/-}$ mice were administered three consecutive daily doses of 60 mg/kg streptozotocin. Non-fasting blood glucose over the following 10 days is displayed. At day 10, grafts were explanted and blood glucose monitored for a further 48 hours to confirm hyperglycemia.

Figure 7-4 Histological analysis of representative graft sections



H&E staining was performed on shipped cells and explanted grafts 8 weeks and one year after cell implantation (top panels). Immunohistochemical analysis was performed on the same sections for insulin, glucagon (Gluc), somatostatin (SS) and pancreatic polypeptide (PPP) (middle and lower panels). (k=kidney)

Figure 7-5 Grafts one year after transplantation



Explants of grafts were performed one year after implantation. Photographs of grafts in situ and after nephrectomy are shown in a and b. Representative H&E-stained sections are displayed in c (2.5x), d (50x) and e (100x).

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Chapter 8: Conclusions and Future Directions

Islet Transplantation: An approach to the treatment of diabetes

Diabetes: A worldwide burden

The world health organization estimates that by the year 2030 nearly 370 million people will suffer from diabetes(*1*). While only 10% of this striking number will suffer from type 1 diabetes (~37 million people) these patients have usually suffered since childhood and have needed to alter their lives in order to cope. Insulin, discovered by Drs Banting, Best and Collip in 1921 (*2*) (Figure 8-1), is the mainstay of therapy in these individuals; even with adequate therapy, and despite newer advances including insulin pumps(*3*), type 1 diabetes patients can still suffer from many of the secondary complications of diabetes and life-threatening swings in blood glucose levels(*4*). Alternative treatment methods were sought.

Islet Transplantation: A brief history

In the late 1800's Drs Watson-Williams and Harshant attempted to transplant minced sheep's pancreas into the subcutaneous tissues of a young boy suffering form diabetic ketoacidosis(*5*). While this very early xenotransplant was unsuccessful, the attempt itself should be applauded. The discovery of insulin

many years later led to a widespread euphoria which took at least 20 years to subside once patients began suffering form many irreversible complication of prolonged hyperglycemia. The early success of kidney transplantation in the 1960s parented the birth of transplantation for diabetes as a serious concept. This was followed by the realization that transplantation of only the endocrine portion of the pancreas was necessary to treat diabetes; a concept initiated by the poor initial results of vascularized pancreas transplantation.

The following decades a resurgence of laboratory study in islet transplantation including Paul Lacy's development of a method for rodent islet isolation and the first successful rodent islet transplant (*6*), the description of a density gradient to purify islets by Scharp and colleagues(*7*) and the first attempts at islet implantation into the portal vein(*8*). This was followed by further advances including the Ricordi chamber (*9*), dithizone-staining of islets for quantification (*10*) and islet transplantation into large animal models(*11-13*). The first case of clinical insulin independence (albeit for only one month) in 1990(*14*) set the stage for a busy decade of islet transplantation which culminated in the publication of the Edmonton Protocol in 2000 in which 7/7 patients became insulin independent (*15*).

Islet Transplantation: Current Status and Limitations

There have been an estimated 750 clinical islet transplants worldwide. While the initial results of the Edmonton Protocol were quite promising, the rates of insulin independence decline over time. The latest Clinical Islet Transplant Registry (CITR) data shows that 70% of worldwide islet transplants reach insulin independence, 55% remain insulin independent two years after islet infusion(*16*) and only 15% remain so after five years(*17*). While the majority of these patients do retain C-peptide positivity, they have required multiple (at least 2-3) islet infusions to reach this point. Initial islet loss and failure of engraftment certainly play a role in the necessity for a large transplanted islet mass; up to 60% of transplanted islets can be lost in the first two weeks(*18*). The decline in islet mass over time is likely multifactorial with auto- and alloimmunity in addition to non-immunological factors all contributing. Newer islet transplant protocols have allowed transplantation of fewer islets, some have even achieved insulin independence with islets from single donors(*19, 20*). Even newer, unpublished reports have pointed towards rates of insulin independence reaching those achieved with pancreas transplantation; even out to three years after transplantation. That being said, islet transplantation has its limitations which are preventing its more widespread application.

Limitation 1: Lack of donor tissue

Current islet transplant protocols utilize pancreatic tissue from deceased human donors. There are only an estimated 13 organ donors per million people in Canada, slightly more (20 per million) in the United States. This is certainly far below the number needed to treat the nearly 300,000 patients with type 1 diabetes in Canada. This discrepancy is even wider considering that most islet transplant recipients require more than one islet infusion. Improving organ donation rates and allocation, finding alternative sources of insulin-producing tissue (eg. stem cells) or discovering ways to use fewer islets (eg enhance engraftment) with improved outcomes would all go far to overcoming this limitation.

Limitation 2: Large islet mass required to gain insulin independence

One of the key findings of the Edmonton Protocol was that transplantation of an adequate combined islet mass was necessary to achieve insulin independence (> 10,000 islet equivalents per kg recipient weight)(*15*). Although some sites have utilized single donors (~7,000 IE/kg) to achieve insulin independence, this is not universal(*19*). Requiring a larger islet mass puts pressure on the islet procurement and isolation process since higher islet yields are required for release of islet preps for transplantation. Finding ways of reducing this mass and making single donor transplants a widespread reality would open up islet transplantation to a wider patient base. The early loss of islets during the

engraftment phase is one of the main factors which necessitates the infusion of a large mass. While improvements in islet procurement, isolation and culture would help reduce the required islet mass, a reduction in early post-transplant islet loss may have an even bigger benefit.

Limitation 3: Risks of the procedure and treatment protocol

The islet implantation procedure itself has its risks. While percutaneous infusion of islets into the portal system obviates the need for a laparotomy and its associated risks, it is not without complications. Post-infusion life-threatening bleeding, portal vein thrombosis and bile leakage are all possible(*21*). Alterations in the protocol, including the use of Avitene paste to seal the catheter tract and post-procedure anticoagulation have helped to reduce these risks(*22*). The portal vein site has become the standard for islet transplantation. The relative ease of access and physiologic insulin release have allowed islet transplantation to reach its current success. However, it has become difficult to monitor islets post-transplant and determine the direct effects of immunomodulatory or engraftment-enhancing agents.

Unlike hepatic or renal failure, diabetes is not necessarily a life-threatening condition. As such lifelong immunosuppression, and the associated side-effects, are easier to justify for solid organ transplantation. This necessitates strict inclusion criteria for patients to be considered for islet transplantation (Table with inclusion criteria). The original Edmonton Protocol immunosuppressive protocol

(Daclizumab, Sirolimus and Tacrolimus) was an improvement; corticosteroids and their inherent islet- and patient-toxic effects were removed(*15*). More recent protocols are now moving away from Sirolimus since it can cause 1) reduced islet engraftment, regeneration and functional survival(*23-25*) 2) intolerable mouth ulcers and neutropenia and 3) worsening renal function in combination with Tacrolimus(*26, 27*). Efalizumab (although now off the market), alemtuzumab and mycophenolate mofetil have all been employed in protocols with enhanced success being observed with the latter two at the University of Alberta.

Safer immunosuppressive agents and even less islet-toxic ones would help to broaden the scope of patients eligible for this procedure. While immunosuppressive agents are not the focus of this thesis, there are ways in which the compounds explored herein could improve outcomes in this field. This will be discussed below.

Dissertation, Findings and Implications

The aim of this thesis is two-fold 1) to explore means to enhance the engraftment of islets and 2) to consider stem cells as a replacement for islet cell transplantation.

Enhancing Islet Engraftment

Engraftment is a crucial stage in the post-transplant life of an islet. It is during this time that they are prone to destruction through apoptosis or necrosis. We have presented strategies in this thesis to enhance islet survival and prevent islet loss during this pivotal time. Although the ideas and interventions seem unrelated, they act on closely related pathways as shown in figure 8-2.

Caspase inhibition

Apoptosis is the main mode of islet loss in the early post-transplant period, accounting for up to 60% of islet loss during the first few days following implantation (*28*). At the heart of apoptosis are the caspases, serine proteases activated in a hierarchical order culminating in the degradation of intracellular protein substrates. This leads to the cellular changes associated with apoptosis including chromatin condensation, nuclear degeneration and cellular dehydration(*29*). The signals leading to β-cell caspase activation can be either

extrinsic (Fas, Cytokines) or Intrinsic (hypoxia, reactive oxygen species, DNA damage).

In chapters 3 and 4 we utilized the capsase inhibitor IDN6556 (Conatus Pharmaceuticals, formerly PF3491390, Pfizer)) in first a mouse model and then a large animal model. Although we did not compare this compound directly to zVAD-FMK or EP1013, it displayed similar efficacy in enhancing islet engraftment and islet survival *in vivo* in our marginal islet mass mouse model. We showed a reduction in early post-transplant apoptosis which translated into improved graft metabolic function.

Although we did not utilize oral drug dosing or the portal vein transplant site, the positive results in the mouse study still have a dual impact: 1) reaffirm “proof of principle” of post-transplant caspase inhibition as an effective means to enhance islet engraftment and 2) allow us to further evaluate IDN6556 in a large animal model. In chapter 4, using a porcine model of diabetes and oral administration of IDN6556 we found a significant improvement in post-transplant glycemia and metabolic function. In summary, these two chapters provide evidence for the effectiveness of IDN6556 in enhancing islet engraftment and survival through a reduction in apoptosis.

Should we be inhibiting apoptosis? One could argue that islets which receive a signal to undergo apoptosis are damaged or have been negatively affected. These islets, if saved from apoptosis by a caspase inhibitor, may be functionally incompetent and may detrimentally affect surrounding islets. Our results would dispute this since grafts in animals receiving the caspase inhibitor display improved metabolic function and enhanced survival (as measured by insulin content in the mouse model). Furthermore, as detailed previously, there are multiple forms of cell death including ones which occur without caspase activation(*30*). Could islets that are prevented from entering apoptosis instead undergo another form of cell death? This is certainly possible, with necroptosis via the mitochondria being a possible alternative(*30*). We did not specifically assess for other forms of cell death, a possible limitation of the included studies. That being said, the inhibition of apoptosis provided a benefit to engrafting islets and it may be that this form of cell loss dominates in the early post-transplant period.

We designed our experiments so that caspase inhibition is attempted during the time of maximal islet engraftment (~ 7 days). While longer dosing periods may be more effective, it is possible that this may also increase the likelihood of negative side effects. Malignancy is a realistic concern with caspase inhibition since apoptosis is a natural mechanism to control cell quality and quantity. In our mouse and pig models we did not observe evidence of malignancy. In a phase II trial, utilizing doses upwards of 100 mg orally for two weeks, there were no

observed side-effects in the 35 days of follow-up(*31*) while a second study utilizing IDN6556 in liver transplant recipients showed no adverse events out to one year(*32*).

The observations of caspase inhibitor effectiveness in this thesis have been made in settings free of alloimmunity. There are no guarantees that these results will translate to the clinic where both allo and autoimmunity pay large roles in islet loss. It is also unclear how caspase inhibitors will interact with the cocktail of immunosuppressive drugs routinely given to patients. A study in liver transplant recipients receiving IDN6556, showing no apparent issues with drug interaction, sheds some light on this second point(*32*). Emamaullee et al added caspase inhibition to co-stimulatory blockade in mice receiving fully-mismatched islet grafts. There was a significant increase in graft survival in mice receiving both compounds as compared to co-stimulatory blockade alone(*33*). A reduction in early islet loss could lead to a smaller antigen load form dying β-cells and less stimulation of incoming immune cells. This points towards caspase inhibitors as being beneficial in the clinical allotransplant setting.

Anakinra and etanercept

The use of peri-transplant anti-inflammatory agents is not a new concept in clinical islet transplantation including ASA, non-steroidal anti-inflammatory drugs (NSAIDs) multivitamins, pentoxiphylline and newer agents such as anakinra (IL-1 receptor antagonist), infliximab (monoclonal antibody to tumor necrosis factor

alpha) and etanercept (dimeric fusion protein consisting of Fc portion of IgG and TNFalpha receptor). Etanercept has found its way into peri-transplant cocktails at a number of transplant centers including Minnesota(19) and Chicago(20). In fact, the recent success of single-donor islet transplantation has been partially attributed to this compound(19). Its initial use in the clinical islet transplant realm was based on a single study using a mouse syngeneic islet transplant model (*34*). The combination of etanercept and anakinra has also been used recently although results are difficult to interpret due to low numbers (n=3) (*35*). The aim of chapter 5 is to provide further evidence for the use of etanercept-alone and study the dual anti-inflammatory cocktail of etanercept and anakinra employing human islets in an immunodeficicent mouse model.

The combination of anakinra and etanercept proved the most efficacious in our human islet transplant model yielding significantly increased diabetes reversal, metabolic function and graft survival. Mechanistically, we displayed a reduction in graft apoptosis in the very early post-transplant period. While this helps to explain the effect of this dual-therapy, we did not search for other mechanisms which may have been present. We did not observe a beneficial metabolic effect of either compound alone although they did reduce the post-transplant graft apoptosis versus control.

Clinically, this points towards the use of anakinra and etanercept in combination to help enhance post-transplant islet engraftment. Care must be taken though as

a statement by health Canada has relayed an increased risk of infection when these compounds are taken concurrently (*36*). The recent clinical report of enhanced engraftment and reduced transplanted islet mass in three patients did not report an increased risk of infection (*35*). Islet transplant recipients are given prophylactic broad spectrum antibiotics in the peri-transplant period. These may need to be continued while patients receive the anakinra/etanercept cocktail and additional agents (eg antifungals) could be added. Should etanercept (without anakinra) be removed from current islet transplantation protocols based on our results? While our study failed to show a metabolic benefit to single-etanercept therapy, there was a benefit to post-transplant apoptosis. Alternative doses may have provided enhanced metabolic benefit, although we did not explore these. The side-effects of etanercept-alone are reported to be very low, with infections and malignancy showing the highest incidences (*37, 38*). As such the potential benefits of etanercept, since it is regarded as one of the reasons for recent single-donor success, may outweigh the risks.

Stem cells in the treatment of type 1 diabetes

Chapters 7 and 8 focus on the use of stem cells to treat type 1 diabetes. As discussed above, one of the limitations of islet transplantation is the relative lack of donor tissue. This could be circumvented with the use of an alternative source of insulin-producing cells. Living-donor islet transplantation has been performed once but has certainly not seen wide acceptance (*39*). This procedure puts the

donor at risk of impaired glucose tolerance after partial pancreatectomy and there is certainly no guarantee that the recipient will become insulin independent. Xenotransplantation, using neonatal porcine islets, is promising since pig insulin is functional in humans and donors are readily available. Several small clinical trials have been conducted with very few cases of long-term insulin independence(*40, 41*) although early success has been observed in non-human primate models(*42, 43*). One concern about this technique is the possible transmission of a zoonotic infection from pigs to humans. Research still continues, including the use of co-stimulation blockade to enhance outcomes(*44, 45*).

Stem cells are another possibility to overcome the lack of donor cells. Chapter 6 introduced the potential sources of stem cells and some of the risks associated with them. Their main advantage is an almost limitless source of cells, provided the correct differentiation and proliferation signals. In chapter 7, we tested the ability of human embryonic stem cells, differentiated to pancreatic endoderm, to further develop and provide metabolic function in an immunodeficient mouse model. In fact we further proved that shipment of these cells (up to 48 hours), does not alter their capacity to function once implanted at the receiving site. The ability of implanted and differentiated cells to provide metabolic control in a glucose-dependent manner is a step towards stem cells becoming a realistic treatment for diabetes. However, as we discovered, this area must be

approached with caution as there are potential downsides including unregulated proliferation leading to teratoma formation. This will be discussed further below.

Future Directions

The Future of Islet Engraftment

Treatment of donors

Our islet engraftment models have focused on treatment of islet recipients during the first 7-10 days following transplantation. It is likely that islet detriment, mainly apoptosis, begins in the donor and continues throughout the islet procurement and isolation process (*46, 47*). Should we consider treatment of the donor prior to pancreas retrieval? A study by Takahashi et al sheds light on this (*48*). Islet-donor mice were treated with dehydroxymethylepoxyquinomicin (DHMEQ), an inhibitor of NF-κB (a key player in islet apoptosis, Figure 8-2), two hours prior to islet procurement. DHMEQ inhibited NF-κB activation, reduced islet apoptosis and improved islet engraftment in a syngeneic islet transplant model. This raises the possibility that treating a donor with a supra-therapeutic dose of a caspase inhibitor may improve post-transplant outcomes. Clinically, one would need to ensure that administration of an anti-apoptotic agent would not prove detrimental to the harvested organs, since it is likely that large doses would be required. However, apoptosis is a detrimental factor in other organ systems (including lung(*49*) and liver(*32*) and so caspase inhibition may prove universally effective in organ procurement and transplantation.

This could be initially explored in a mouse model similar to the Takahashi study; dosing of donor mice with IDN6556 prior to pancreatectomy. Serum from donors should be collected to assess for damage to viscera including liver (liver enzymes) and kidneys (creatinine, urea). Islets would then be transplanted into diabetic syngeneic recipients. Once could then explore the effect of addition of IDN6556 to isolation reagents and media; exposing islets to caspase inhibtion throughout. In chapter 3 we explored the effect of co-culture of human islets with IDN-6556. The observed benefit in islet survival points toward inclusion of IDN6556 in islet isolation reagents as a worthwhile endeavour.

Monitoring Engraftment

In this thesis we have studied the effects of compounds in islet engraftment using diabetes reversal, metabolic function and graft survival as markers of effectiveness. Diabetic mice are followed for return of euglycemia as transplanted islets engraft and function. Those mice with grafts which fail to engraft are euthanized in a human manner. In the clinic, patients are transplanted with subsequent islet infusions if the initial ones fail to provide adequate function. Following engraftment in real-time is very difficult. Obtaining liver biopsies is potentially dangerous and only successful in visualizing islets in 31% of cases(*50*). Additionally, preparation of histology is time consuming and may not provide answers in the very early engraftment period (minutes to hours). Following markers of islet injury or damage, including insulin mRNA release, is possible but does not provide a visual cue or is it sensitive enough to detect early

events(*51*). In fact, the release of c-peptide and the increase in coagulation parameters during the first 15 minutes following islet infusion indicates that there is islet injury even this early(*52*).

Visualization of the islet graft post-transplant with positron emission tomography (PET) is one possible solution. PET involves the use of the tracer 2-deoxy-2[^{18}F] fluoro-D-glucose ([^{18}F]FDG) which is taken up into cells on the same transporter as glucose. This technique is used in the field of oncology to find tumor metastases, since these cells have a rapid growth rate and enhanced glucose uptake. Islets could be labeled with [^{18}F]FDG prior to transplantation and then visualized post-transplant using PET. This is exactly what Eich et al did in a large animal (pig) model(*53*). Uptake of [^{18}F]FDG was observed in islets *in vitro* which was also observed immediately post-transplant. One observation was that only 50% of the infused radioactivity was observed within the liver. It is hypothesized that the remaining 50% was contained in islets which are severely damaged upon infusion and their contained tracer released. This aside, this is a potentially useful technique to assess early post-transplant engraftment. The effect of various agents on engraftment could be assessed rapidly with further optimization of dose and timing in large animal models before application to the clinic. In fact, this technique could readily be applied in the clinic; enabling rapid determination of treatment effect.

The transplantation of islets into a more readily accessible site could facilitate graft monitoring. The intramuscular site has been of particular interest for these reasons. Three diabetic human kidney transplant recipients had a subtherapeutic dose of islet placed in their forearm(*54*). On biopsy, two of the three grafts showed β-cell staining and an infiltrate consistent with autoimmune disease. The group in Sweden transplanted a 7-year-old girl with autologous islets (6400 islets/kg) after total pancreatectomy(*55*). At 27 months of follow-up she was gaining weight, had a normal HbA1c, a low insulin requirement and the absence of recurrent hypoglycemia. In mice, intramuscular islets displayed enhanced revascularization with vessel densities and blood flow comparable to the native pancreas(*56*). Islets could be assessed post-transplant using magnetic resonance imaging (MRI). This technique could be combined with an MRI contrast agent in order to enhance visualization(*57*). Even if the full islet mass is not transplanted in the muscle, a small “sentinel” graft could be placed at this site to facilitate monitoring of the larger mass placed intraportally.

The future of stem cells as a treatment for diabetes

The future of stem cells in diabetes therapy is not clear at this point. There are multiple sources for stem cells, each with its own advantages and disadvantages. No matter what cellular strategy is utilized to provide pancreatic endocrine function, the autoimmune insults which caused the initial disease are still present

in the recipient. This autoimmunity is likely to attack the transplanted cells, even if they are derived in an autologous manner.

Induced pluripotent stem cells (iPSCs)

Induced pluripotent stem cells are cells derived from somatic tissues and induced to a more pluripotnet state through “forced” expression of certain genes. One advantage of these cells is that they could be derived form a patients own cell population and avoid the ethical dilemma of using human embryonic stem cells. Takahashi and Yamanaka found that retroviral expression of a set of genes (*Oct4, Soc2, Klf4 and c-Myc*) converted somatic cells into a pluripotent state, although at a low efficiency (*58*). These results have been subsequently repeated with human cells (*59*) and virus/vector-free (*60, 61*). These cells have the potential to provide scientists the tools to test new drugs or treatment strategies and enable autologous cell transplantation for clinical therapy (Figure 8-3) This later option has the potential to provide a large boost to diabetes therapy; harvesting a patient’s own cells, generating pluripotent cells from them and then differentiating them into insulin-producing cells before autologous transplantation. Maehr et al under the guidance of Douglas Melton have developed insulin-producing glucose-responsive cells from patients with type 1 diabetes (*62*). Transition to the clinic will be much more challenging as evidenced by a recent attempt in the field of spinal cord injury and the subsequent FDA concerns regarding tumor formation (*63*).

Teratoma formation and risk

Teratomas are a substantial risk for any pluripotent stem-cell-based therapy including iPSCs and hESCs. Teratomas are encapsulated tumors with cells derived from all three germ layers. The risks of this tumor are twofold: 1) The continued growth and space occupation and 2) the very rare potential of malignant transformation. While most teratomas can be surgically excised, there is certainly very little justification to cure a patient's diabetes with stem cells only to develop a troublesome tumor.

The risk of teratoma formation is certainly heightened in an immunosuppressive environment (eg immunodeficient mouse model) where the frequency of tumor formation is directly related to the degree of immunosuppression (*64*). In chapter 8 we discovered 100% teratoma formation when hESCs were implanted into immunodeficient mice. Of note, sirolimus may have the opposite effect. It is known to possess anti-proliferative and anti-tumor effects and may prevent teratoma formation(*65-67*). This should be studied in a mouse model such as the one we have presented here. It is unclear if transplantation of iPSCs into a genetically matched individual will have the same response; although it is entirely possible that the rate of formation will be enhanced in the absence of an immune response.

While surgical resection of a teratoma is certainly one option, preventing its formation is certainly a preferred option. Transdifferentiation of one cell type into another would circumvent the need for a pluripotent cellular population and the potential for teratoma formation (*68, 69*). One limit of these cells is that they would not be expandable and lack the limitless growth of hESCs/iPSCs. In is possible that encapsulation devices (eg the Theracyte device(*70, 71*)) could provide a physical barrier to teratoma formation, although this has not been proven. Removal of residual fully pluripotent cells from iPSCs (eg using cell sorting) is certainly possible in theory but unproven to eliminate the teratoma risk.

Islet Transplantation – The Past Decade, The Next Decade

It has now been a decade since publication of the Edmonton Protocol (*15*). The remarkable results in these seven patients renewed interest in the field of islet transplantation both clinically and experimentally.

The last ten years

How much progress has actually been made in the past ten years? The five-year follow up after the original Edmonton publication revealed that insulin independence rates drop to nearly 10% over this time period even though c-peptide is present in the majority(*17*). While this series only included patients from Edmonton, a further international trial proved that the initial Edmonton Protocol results could indeed be replicated elsewhere(*72*). Current results presented at the International Pancreas and Islet Transplant Association (June 2011) hint at three-year insulin independence rates nearing those of pancreas transplantation (~80%). However, this is usually only possible in centers with the highest islet transplant experience. Remarkably, high rates of insulin independence are now possible using single donor marginal mass islet infusions (*19*). To obtain these improved results, groups have employed newer, and less islet-toxic agents in their peri-transplant immunosuppression protocols. Etanercept, alemtuzumab (Campath, anti-CD52) and mycophenolate mofetil (an

inhibitor of purine biosynthesis) are all used in various protocols. These target unique and varied aspects of the post-transplant immune and inflammatory attack on freshly transplanted islets. It is difficult to determine the exact contribution of each of these agents since none have been investigated in a randomized trial of islet transplantation. However, improved results have made it difficult to move away from these newer agents with the further addition of compounds based on pre-clinical and/or theoretical benefit (eg. etanercept).

The islet transplant procedure itself has remained relatively unchanged. The majority of islet infusions occur percutaneously into the portal vein. Early complications including post-procedure bleeding and thrombosis have been nearly abolished(*21*). The use of Avitene® paste to plug the catheter tract eliminated the former while heparin anticoagulation and careful imaging follow-up reduced the latter. Although the portal site has its drawbacks (IBMIR, difficult islet monitoring etc.) it has remained the only site utilized routinely. Alternative sites are constantly being explored but have thus far failed to supplant the portal site. Intramuscular(*55*), gastric submucosal(*73*) and bone marrow(*74*) sites have all been tested pre-clinically although progression to the clinic has been slow. Certainly, if a new site were to be applied clinically it would need to display improved outcomes or a significant advantage (eg. enhanced graft monitoring) over the portal site.

The next ten years

Islet transplantation from deceased donors remains a promising treatment for brittle type 1 diabetic patients. However, this is not the only potential source of insulin. As this thesis has shown, stem cells could replace islet transplantation in this patient population. In addition xenotransplantation, using neonatal porcine islets remains a topic of considerable research interest. Will either of these sources replace islet transplantation in the next decade?

While this may seem like a certainty to some, I believe the near future lies with islet transplantation. Xenotransplantation, while a promising treatment in theory, has only achieved moderate progression in results. Some of the most promising results include an average of 60 day rejection-free survival of neonatal porcine islet grafts in non-human primates(*75*). Overcoming the potent immunosuppressive barrier of xenotransplantation is a difficult task; one that may not be overcome without genetic engineering of islets to reduce the xenogenic stimulus.

Stem cells, although potentially able to provide a limitless source of insulin, are similarly a number of years away from clinical utilization. While pre-clinical small animal studies have been promising, displaying in vivo function of both human and rodent-derived stem cells, there have been no studies in large animal models or in humans. Should stem cells be transplanted into the portal vein or

will a more readily accessible site (eg. subcutaneous) provide a safer alternative? What is the long-term function of stem cells and what are their side effects? We have shown that teratoma formation is certainly one detrimental effect, although there may be others. Certainly there are a number of questions remaining before stem cells see their place in the treatment of clinical diabetes.

While alternative sources of insulin are still years away from clinical application, research should continue on islet transplantation. It is possible that results discovered while studying islets could be applied to alternative therapies. This could include alternative transplant sites, encapsulation devices, more potent and safer immunosuppression protocols, imaging modalities and further methods to enhance islet survival.

Overall Conclusions

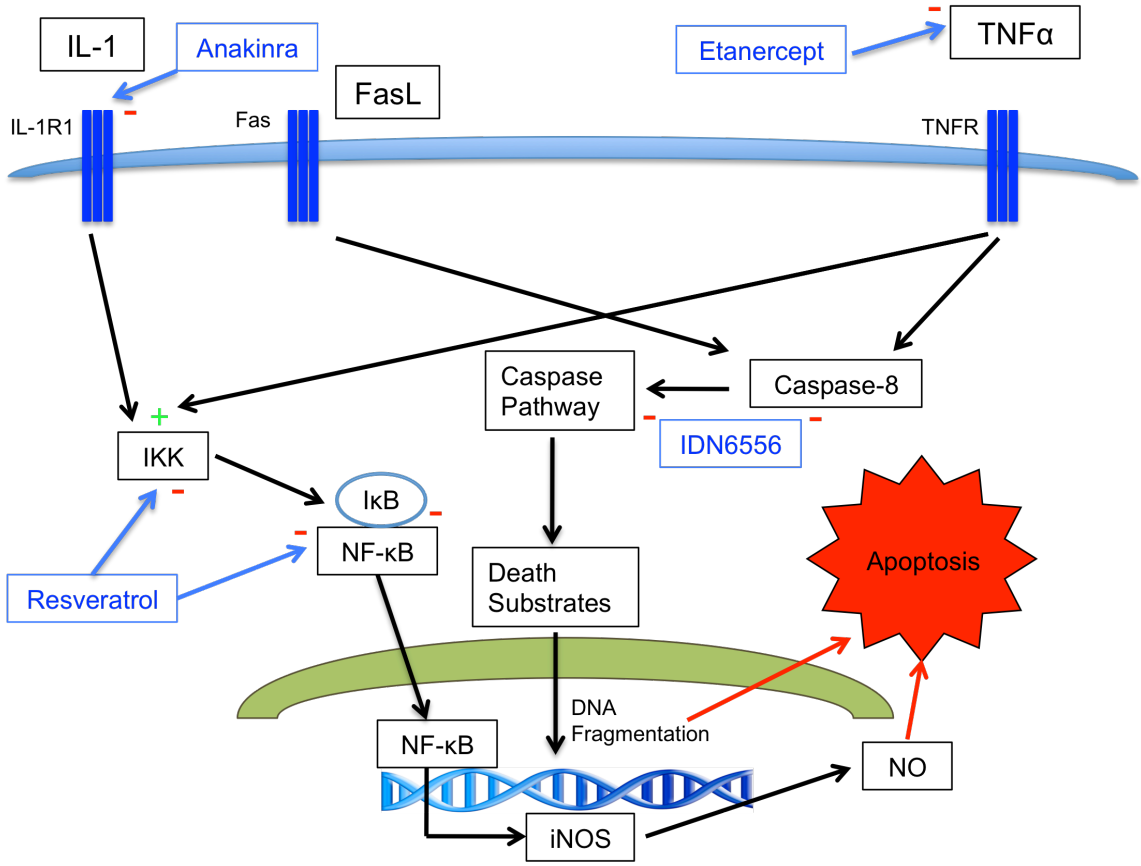
Islet transplantation has become a very promising treatment for select patients with type 1 diabetes. However, it does have its limitations including the loss of a large proportion of islet mass after infusion and a limited source of tissue.

Preventing islet apoptosis with caspase inhibitors or agents targeting cytokines has the ability to enhance islet engraftment and preserve the transplanted islet mass. This leads to improved metabolic performance and islet survival. Application in the clinic could reduce the islet mass necessary for insulin independence which would allow more islet preparations to reach the minimum islet mass for clinical release. We could potentially reach a state where multiple patients are being treated with each harvested pancreas. While this may be optimistic, the hope of longer-term insulin independence is certainly promising with these treatments.

Finding an alternative source of insulin-producing tissue would overcome the need to rely on cadaveric organs and may allow a broader patient population to be treated. While there are still a number of issues to address including long-term risks and ethical debates, we have demonstrated reproducible *in vivo* metabolic function of one cell line, bringing us even closer to a seeming eventuality.

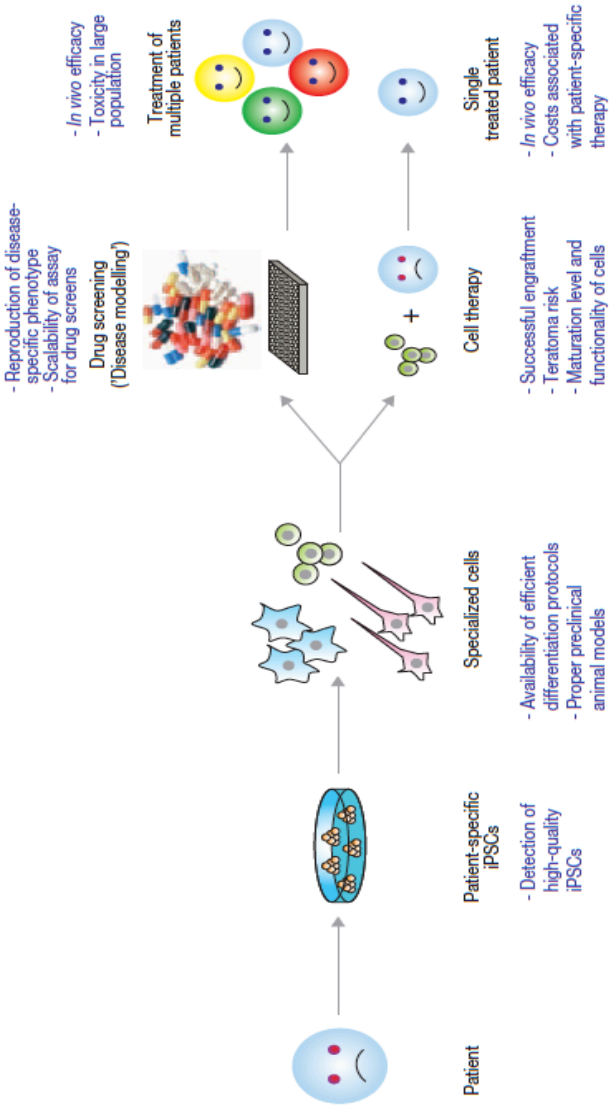
Figure 8-1 Drs Banting and Best pose with one of their research animals

Figure 8-2 Pathways to beta cell apoptosis and how the interventions presented in this thesis may interact with them



Fas/FasL Fas/Fas ligand
IκB Inhibitor of NF-κB
IKK IκB kinase
iNOS Inducible nitric oxide synthase
IL-1 Interleukin 1
TNFα Tumor necrosis factor alpha
NO Nitric oxide
NF-κB Nuclear factor kappa-light-chain-enhancer of activated B cells

Figure 8-3 Potential strategies in the use of induced pluripotent stem cells to treat disease



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Appendices

Preface to Appendix 1

Resveratrol, a polyphenol found in fruits and vegetables, has been heralded as having **anti-inflammatory**, **anti-oxidant** and even **anti-aging** properties. In addition to its cardioprotective benefits, pre-clinical rodent studies have shown resveratrol to possess the ability to **lower blood glucose** and **prevent islet cytotoxicity**. However, no studies have yet explored the effects of resveratrol on islet transplantation and specifically islet engraftment.

Resveratrol fails to improve marginal mass engraftment of transplanted islets of Langerhans in Mice

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RESEARCH PAPER

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Resveratrol fails to improve marginal mass engraftment of transplanted islets of Langerhans in mice

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Key words: resveratrol, islet transplantation, engraftment, mouse, islets

Abbreviations: AUC, area under the curve; CMRL, connaught medical research laboratories; ELISA, enzyme-linked immunosorbent assay; HBSS, hank's buffered salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IκB, inhibitor of NFκB; IKK, inhibitor of IκB kinase; IL-1, interleukin-1; INFγ, interferonγ; iNOS, inducible nitric oxide synthase; i.p, intraperitoneal; IPGTT, intraperitoneal glucose tolerance test; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; PI3K, phosphoinositide-3-kinase; TNFα, tumor necrosis factor alpha

One limitation of current islet transplantation protocols is the loss of up to 70% of the transplanted islet mass. Inflammatory events play a major role in islet loss including the cytokines TNFα and IL-1. Resveratrol, a compound with anti-inflammatory and anti-oxidant properties, has the potential to mitigate islet loss. Using a syngeneic marginal after mouse islet transplantation model we tested the ability of resveratrol to enhance islet engraftment. We failed to show a difference in diabetes reversal between mice treated with vehicle and those treated with either 10 mg/kg (47.1% for resveratrol vs. 35.3% for control) or 50 mg/kg (20% for resveratrol vs. 22.2% for control) of resveratrol daily for three weeks. In addition, at one month there was no difference in glucose tolerance or graft survival (10 mg/kg: 552.6 ng/ml resveratrol group vs. 576.6 ng/ml control group; 50 mg/kg: 463 ng/ml resveratrol group vs. 444.1 ng/ml control group). In summary, over a wide range of doses, resveratrol did not exert a benefit on mouse islet engraftment. Further studies should be conducted with human islets before deeming resveratrol ineffective in islet engraftment and survival.

Introduction

Type 1 diabetes is a disease projected to affect at least 366 million people worldwide by the year 2030.[1] Autoimmune destruction of β-cells leads to deficient insulin production and hyperglycemia, requiring lifelong insulin injections. For a subset of patients with type 1 diabetes, islet transplantation may be an option to replace the lost β-cell mass.

In 2000, outcomes from clinical islet transplantation improved with the Edmonton Protocol, where all seven patients became insulin independent after receiving intraportal islet transplants from at least two donors.[2] An international trial followed with reproduction of these initial results in the most experienced centers.[3] Unfortunately, the majority of recipients needed to return to insulin with only 15% remaining insulin independent five years after transplantation even though the majority (over 70%) demonstrated persistent C-peptide reserve, correction of HbA1C and protection from hypoglycemia. Further refinements of the islet transplantation process and immunosuppressive protocol have led to improvements in islet survival and function. For example, it is now possible to achieve insulin independence with islets from a single donor pancreas.[4,5] However, we are still far from achieving the goal of long term insulin independence for the majority of islet transplant recipients.

Freshly transplanted islets face a number of insults which contribute to their early loss. Hypoxia, allo- and autoimmune reactivity and inflammation all play roles in this. It has been estimated that up to 60% of the transplanted islet mass undergoes apoptosis in the first several days after transplantation.[6] This points to inflammatory events as playing a major role in islet loss. Interleukin 1β (IL-1β), tumor necrosisα (TNFα) and interferonγ (IFNγ) are all cytokines known to contribute to inflammation and islet death.[7-9] Their mechanism of action hinges around the induction of the transcription factor NFκB and the subsequent expression of inducible nitric oxide synthase (iNOS). This results in the production of the toxic free radical NO and β-cell apoptosis.[10] Finding a means of preventing this free radical production and apoptosis could potentially lead to improved early islet survival and, in turn, long term survival and function.

Resveratrol (trans-3,4',5-trihydroxystilbene) is a polyphenol found in fruits and vegetables; especially grapes. It has recently emerged as a promising treatment for a number of diseases

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Resveratrol (trans-3,4',5-trihydroxystilbene) is a polyphenol found in fruits and vegetables; especially grapes. It has recently emerged as a promising treatment for a number of diseases including cancer(11), cardiovascular disease(12), stroke(13) and both type 2(14) and type 1 diabetes. Resveratrol is an activator of the nicotinamide adenine dinucleotide-dependent deacetylase SIRT1(15, 16). Overexpression of SIRT1 can protect β-cells from cytokine toxicity through suppression of the NF-κB pathway(17). It is also likely that resveratrol can directly inhibit NF-κB through the suppression of the inhibitor of NFκB (IκB) kinase (IKK)-mediated phosphorlylation of NF-κB(18). Some argue that resveratrol is not a direct activator of SIRT1(19) and in fact the main activity of resveratrol is inactivation of other proteins including NF-κB and phosphoinositide 3-kinase (PI3K). Regardless, the inhibition of NF-κB by resveratrol could facilitate

islet engraftment in the early post-transplant period when islets are facing an onslaught of cytokines and inflammation. Other potentially protective effects of resveratrol include its ability to lower blood glucose in diabetic rodent models (20-22) and to reduce insulin release from islets(23-25). This latter phenomenon may provide freshly transplanted islets with a period of “rest” which has been shown to delay the progression of type 2 diabetes(26).

In this study, we hypothesize that post-transplant treatment of diabetic mice receiving marginal mass islet grafts with resveratrol will lead to improved diabetes reversal and islet function.

Materials and Methods

Animals and Reagents

BALB/c mice were obtained from the Jackson Laboratories (Bar Harbor, ME). All animals were cared for according to the guidelines of the Canadian Council on Animal Care, and ethical approval was obtained from the animal welfare committee at the University of Alberta.

All reagents, including resveratrol, were obtained from Sigma Aldrich (Oakville, Ontario, Canada) unless otherwise specified.

Mouse Islet Isolation

Mouse islets were isolated using established protocols with minor modifications(27). In brief, mouse pancreata were digested with collagenase (1.0mg/ml in Hanks' buffered saline solution (HBSS)) and purified with Histopaque-density centrifugation. Handpicked islets were washed with HBSS then placed in short-term culture in Connaught Medical Research Laboratories (CMRL-1066) medium supplemented with 10% fetal bovine serum, L-glutamine (100 mg/l), penicillin (112 kU/l), streptomycin (112 mg/l) and HEPES (25 mmol/l). Islets were cultured for a maximum of two hours before transplantation.

Islet Transplantation

Streptozotocin was administered to recipient mice to induce diabetes (220 mg/kg i.p). Animals were considered diabetic after two consecutive blood glucose measurements ≥ 20 mmol/L using a OneTouch Ultra glucometer (Lifescan Canada, Burnaby, B.C). A marginal mass of 150 islets were implanted into the kidney subcapsular space. Transplant recipients were given a once daily intraperitoneal injection of either vehicle (PBS, 100uL) or resveratrol (10 mg/kg or 50mg/kg) on the day of transplant and daily for the first three weeks post-transplant.

Glucose tolerance tests

Transplanted mice were fasted for 16-20 hours and injected intraperitoneally with 50% dextrose at 2g/kg body weight (intraperitoneal glucose tolerance test, IPGTT). Blood glucose levels were analyzed at baseline, 5, 15, 30, 60, 90 and 120 minutes post-injection.

Graft insulin content

Islet grafts were harvested from the kidney capsule and stored at -80°C until bulk analysis could be performed. Extraction was performed in acid-ethanol by homogenization and ultrasonic cell membrane disruption. Insulin concentration of

the neutralized extract was measured using a commercial ELISA kit (Alpco Diagnostics, Windham, NH).

Statistics

Data was analyzed using GraphPad Prism (Version 5.0b, GraphPad Software Inc., San Diego, CA). P values less than 0.05 were considered statistically significant. Graphical representation of data is represented as mean ± SEM, unless otherwise indicated in the figure legends. Column means were compared using the Mann-Whitney test while survival analysis was carried out using log-rank analysis.

Results

Diabetes Reversal

Diabetic mice were transplanted with a marginal mass of syngeneic islets under the kidney capsule and treated with either 10 mg/kg (N=17) or 50mg/kg (N=17) of resveratrol i.p daily for three weeks. Mice in each group were compared to separate control groups of transplanted mice receiving PBS only (N=15). Blood glucose was monitored with a euglycemia defined as two consecutive non-fasting blood glucose readings < 11mmol/L. There was no difference in non-fasting blood glucose throughout the study between either of the resveratrol groups and the controls (Figure A1-1A and B, first panels). In addition, neither the 10mg/kg dose of resveratrol (47.1% for resveratrol vs 35.3% for control, $p > 0.05$) nor the 50 mg/kg dose (20% for resveratrol vs 22.2% for control, $p > 0.05$) had a significant effect on the percentage of mice achieving euglycemia at any point during the study. No mice died prematurely or became ill during the study.

Glucose tolerance

One month after transplantation, mice were subjected to an intraperitoneal glucose tolerance test after overnight fasting (Figure A1-2A). There was no significant difference in the blood glucose levels during the two hours following the dextrose bolus between resveratrol-treated mice and controls. This equated

into area under the curve (AUC) values which did not differ between groups (Figure A1-2B, $p > 0.05$ for both graphs by t-test).

Graft survival

Forty-eight hours after glucose tolerance testing, mice were sacrificed and their grafts recovered to determine the insulin content (Figure A1-2C). This was used as a surrogate for survival of the graft. Mice receiving either dose of resveratrol showed no significant difference in the insulin content of their grafts as compared to control mice ($p>0.05$ by t-test).

Discussion

In this study we failed to demonstrate any positive impact of resveratrol treatment upon islet engraftment and function in diabetic mice receiving a marginal mass islet grafts. In addition there was no benefit to in vivo islet survival, using graft insulin content as a surrogate marker.

Mice received resveratrol for three weeks following transplantation with a resveratrol-free period of one week before glucose tolerance. We chose this dosing strategy so that the glucose tolerance test results would reflect islet function without direct effect of resveratrol. The dose range of resveratrol was selected based on prior studies and reflected a wide therapeutic range (10-50 mg/kg per day). To put this into perspective, the doses approved by the US Food and Drug Administration for human consumption are approximately 7-15 mg/kg per day. We chose the intraperitoneal route based on a prior study showing enhanced efficacy via this route as compared to oral dosing(18). The “optimal” dose of resveratrol still remains unclear, and this may depend on its intended use. It may be that our doses of resveratrol were insufficient to provide an effect on islet engraftment and *in vivo* function in this model. Doses up to, and including, 100mg/kg have been safely employed in rodents(28).

Islet engraftment, occurring over the first 7-10 days after transplantaiton, is a crucial step in the survival of a transplanted islet. During this period, islets are

subjected to cytokine-mediated destruction in addition to allo and autoimmune cellular attack. In the current study, we employed a syngeneic transplant model to isolate the non-immunological events leading to islet loss. Despite debate on the exact mechanism, resveratrol has the potential to prevent cytokine-mediated cytotoxicity to transplanted islets, based on previous in vitro studies(16, 17). In this study, Resveratrol, at the doses employed, failed to prove effective in enhancing islet survival. We can surmise from this that either the compound proved ineffective in preventing cytokine-mediated islet damage or that there are other forms of islet destruction that dominated.

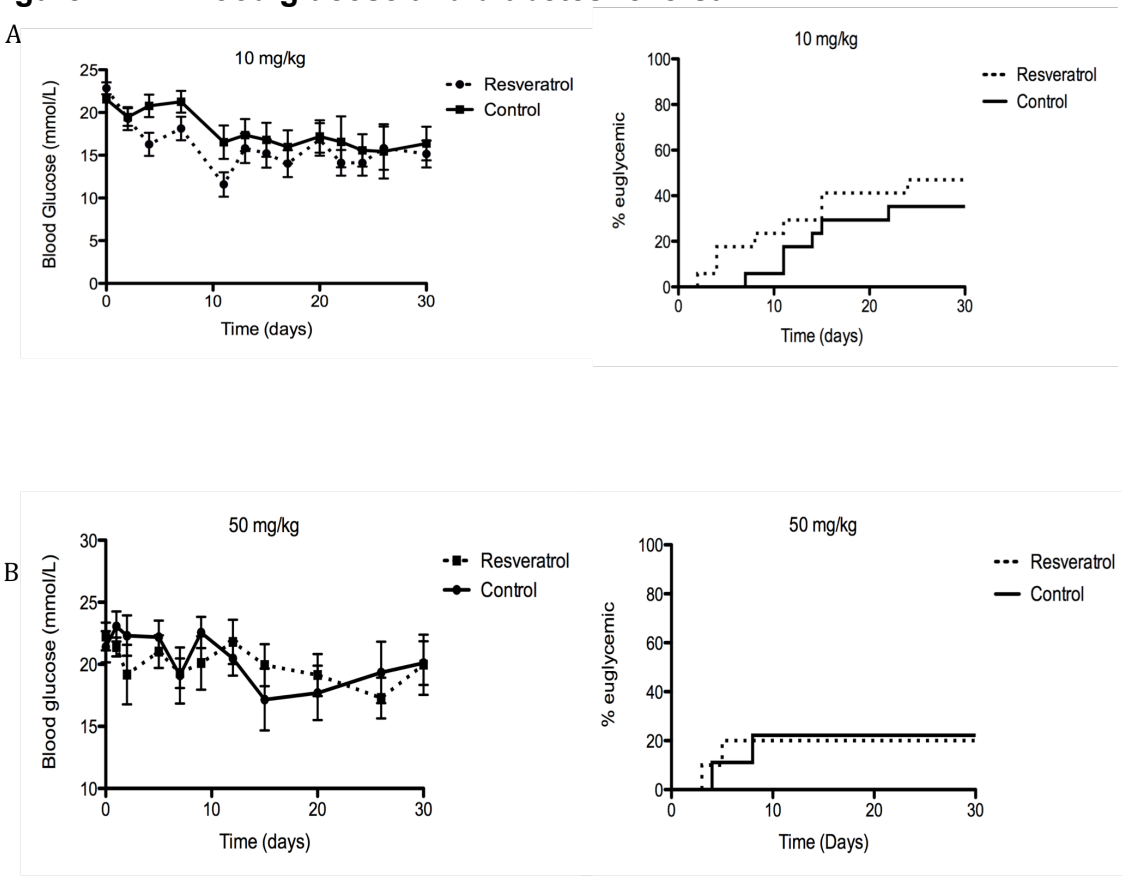
During the three week treatment period, we observed no significant reduction in blood glucose levels in mice treated with resveratrol. These findings are is in contrast to a previous study reporting an insulin-like effect of resveratrol in streptozotocin-treated rats, with a dose of 0.75mg/kg, a much reduced dose compared to our present study in mice(20).

The current results therefore demonstrate no therapeutic benefit of escalating doses of resveratrol in a marginal mass mouse islet transplant model. Since there have been no other publications of outcome specifically of resveratrol in islet transplantation, and yet this compound has been promoted as a universal protector in many areas of medicine, we felt it important to report these negative findings. Limitations of the present study include the lack of a positive control for the functioning of resveratrol and the possibility of positive impact with human

islets, which was not tested in the present study. It could be that mice are resistant to treatment with resveratrol compared with other species, but this is not borne out by several other studies of resveratrol treatment in mouse models of other disease states.

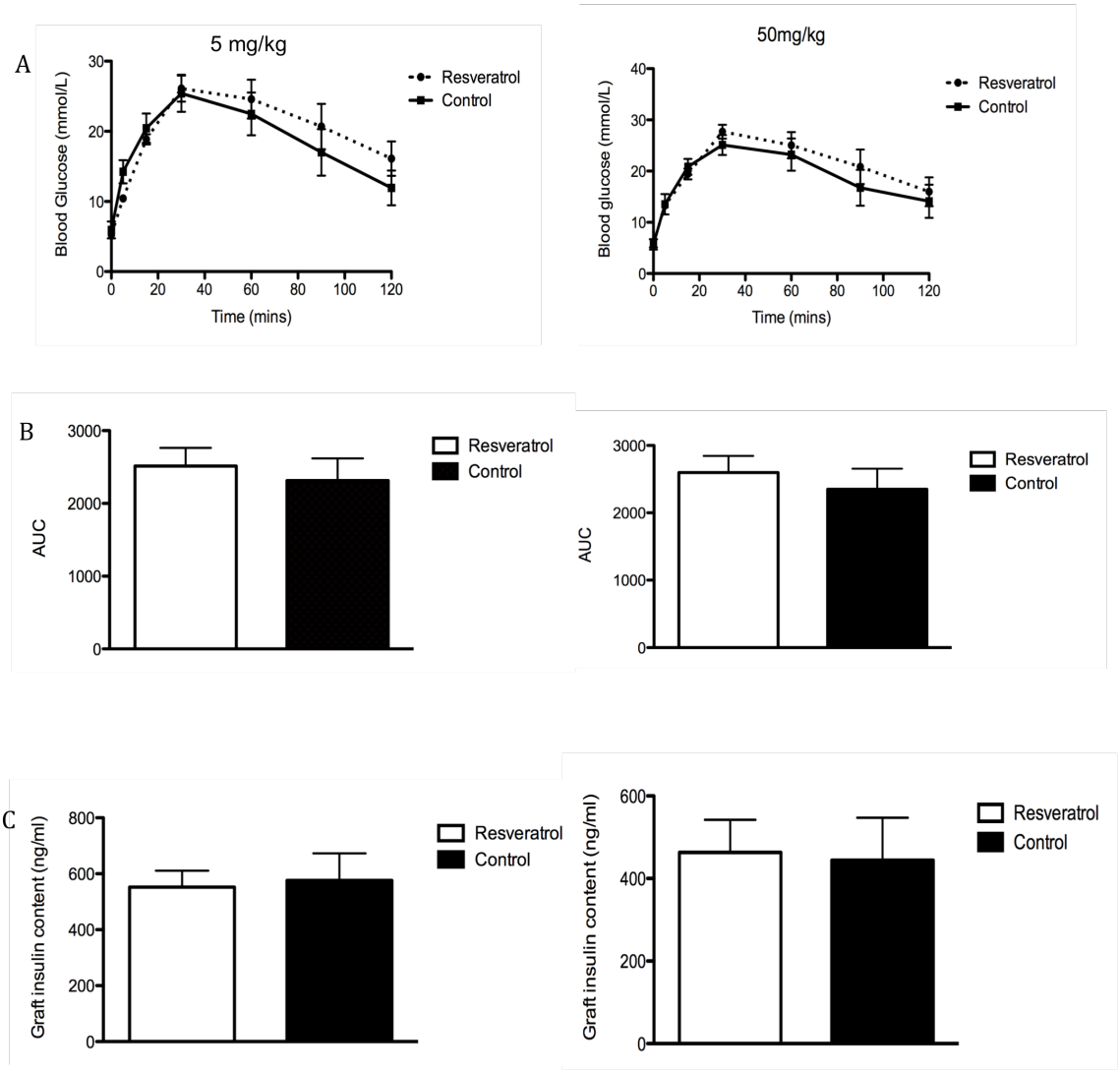
In summary, resveratrol a compound with anti-oxidant and anti-inflammatory properties failed to provide benefit to islet engraftment in a mouse model of diabetes. We also did not observe any benefits to blood glucose over the period of drug administration. It remains unclear if resveratrol will have any benefit in islet transplantation.

Figure A1-1 Blood glucose and diabetes reversal



Diabetic mice received marginal mass syngeneic islet grafts and were treated with either 10 mg/kg (A) or 50 mg/kg (B) resveratrol ip daily for three weeks. In each case, they were compared to a group of mice receiving PBS instead of resveratrol. Non-fasting blood glucose was monitored daily. $p > 0.05$ in % euglycemic graphs by log-rank analysis.

Figure A1-2 Glucose tolerance and islet survival



Mice were subjected to glucose tolerance analysis after a weight-based bolus of dextrose was administered intraperitoneally. Blood glucose was monitored over the following two hours. Area under the curve (AUC) analysis for the curves in A is displayed in B. Dose of resveratrol is displayed. ($p > 0.05$ for both AUC graphs by t-test). Forty-eight hours after glucose tolerance testing, mice were sacrificed and their graft-bearing kidneys recovered for insulin content analysis. After acid-ethanol extraction of insulin, supernatants were analyzed using an ELISA kit. ($p > 0.05$ by t-test for both doses of resveratrol).

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Preface to Appendix 2

Newer and safer immunosuppressive agents are constantly being sought although very few reach the clinical realm. One that has shown promise is AEB-071 (Sotrastaurin), a selective protein kinase C inhibitor. When I started my degree, our lab had published data showing a delay in rat islet allograft rejection using AEB-071 alone and in combination with cyclosporine. In addition, this compound was less islet-toxic than calcineurin inhibitors. Our lab group was invited to prepare a review article on AEB-071, which I wrote with the help of Drs Merani, Toso and Shapiro. This review article is thus included here for completeness of the work I undertook during my research degree.

Sotrastaurin (AEB-071)

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2009 34(8)

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MONOGRAPH

SOTRASTAURIN

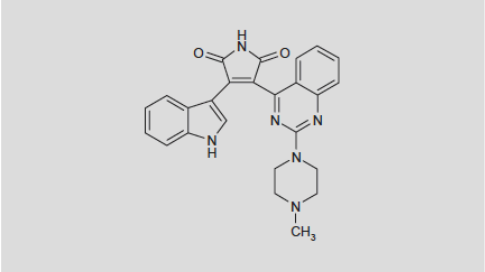
Rec INN; USAN

Protein Kinase C Inhibitor
Treatment of Transplant Rejection
Treatment of Psoriasis
Treatment of Uveitis

AEB-071
NVP-AEB-071

3-(1*H*-Indol-3-yl)-4-[2-(4-methylpiperazin-1-yl)quinazolin-4-yl]-2,5-dihydro-1*H*-pyrrole-2,5-dione

InChI=1S/C25H22N6O2/c1-30-10-12-31(13-11-30)25-27-19-9-5-3-7-16(19)22(28-25)21-20(23(32)29-24(21)33)17-14-26-18-8-4-2-6-15(17)18/h2-9,14,26H,10-13H2,1H3,(H,29,32,33)



$C_{25}H_{22}N_6O_2$
Mol wt: 438.4812
CAS: 425637-18-9
CAS: 908351-31-5 (acetate salt)
EN: 321045

ABSTRACT

Sotrastaurin (AEB-071, NVP-AEB-071) is an orally bioavailable compound that exerts its effects through the selective inhibition of the classic and novel forms of protein kinase C (PKC), thereby inhibiting early T-cell activation and IL-2 production. In preclinical studies, sotrastaurin reduced the rejection of allogeneic solid organ and islet transplants and interacted in a synergistic manner with the immunosuppressive agent ciclosporin. Sotrastaurin is being investigated in a number of clinical trials aimed at exploring its efficacy and safety in T-cell-mediated conditions such as transplant rejection, psoriasis, uveitis and ulcerative colitis. The compound has shown acceptable toxicity profiles in healthy individuals and transplant recipients. Provided sotrastaurin shows continued promise in the ongoing clinical studies, it may be a safe and effective alternative or adjunct to calcineurin inhibitors.

SYNTHESIS**

Chlorination of quinazoline-2,4-dione (I) with $POCl_3$ in the presence of *N,N*-dimethylaniline at reflux affords 2,4-dichloroquinazoline (II). Subsequent condensation of (II) with the sodium salt of ethyl acetoacetate (III) in refluxing toluene/THF followed by deacetylation and amidation in aqueous ammonia leads to 2-(2-chloro-4-quinazolinyl)acetamide (IV). After displacement of the remaining 2-chloride of compound (IV) with *N*-methylpiperazine (V) in NMP at 50 °C, the resulting acetamide (VI) is condensed with methyl 3-indolylglyoxylate (VII) in the presence of *t*-BuOK in THF to give sotrastaurin (1). Scheme 1. The corresponding mesylate and maleate salts are prepared by treatment of sotrastaurin with methanesulfonic acid and maleic acid, respectively, in EtOH at 45 °C (2).

BACKGROUND

Candidate immunosuppressive drugs, although abundant in previous decades, have become increasingly difficult to discover, develop and bring to market. Several recent potential agents did not survive the validation of clinical trials, including fingolimod (FTY-720) (3, 4) and anti-CD154 (5). The prevention of allograft rejection has therefore relied heavily on calcineurin inhibitors (CNIs; tacrolimus, ciclosporin) and mTOR inhibitors (sirolimus, everolimus). These agents, although effective, are associated with a number of toxicities, including nephrotoxicity for CNIs (6) and impaired wound healing and hypercholesterolemia for sirolimus (7). A new agent with an improved side effect profile but at least similar efficacy would therefore have a major impact.

Sotrastaurin (AEB-071, NVP-AEB-071) is a new, orally bioavailable compound that exerts its effects through the selective inhibition of protein kinase C (PKC). PKC isoforms are involved in the activation of T cells downstream of both signal 1 and signal 2 (8). Sotrastaurin

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**Synthesis prepared by R. Pandian, J. Bolós, R. Castañer.
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Background

Candidate immunosuppressive drugs, although abundant in previous decades, have become increasingly difficult to discover, develop and bring to market. Several recent potential agents did not survive the validation of clinical trials including FTY720(1, 2) and anti-CD154(3). Prevention of allograft rejection has therefore relied heavily on calcineurin inhibitors (CNIs, tacrolimus, cyclosporine, CsA) and mTOR inhibitors (sirolimus, everolimus). These agents, although efficacious, are associated with a number of toxicities including nephrotoxicity for CNIs(4) and impaired wound healing and hypercholesterolemia for sirolimus(5). A new agent with an improved side-effect profile, but at least similar efficacy would therefore have a major impact.

AEB-071 (Sotrastaurin, AEB) is a new orally bioavailable compound that exerts its effects through the selective inhibition of Protein Kinase C (PKC). PKC isoforms are involved in the activation of T cells downstream of both signal 1 and signal 2(6). AEB-071 selectively inhibits the classical (α, β) and novel (δ, ε, η, θ) PKC isoforms and thus effectively blocks early T cell activation and subsequent IL-2 production (Figure A2-1). Several studies have suggested that AEB is effective in preventing allograft transplant rejection will provide a better-tolerated alternative to the CNIs. Since pathologic T-cell activation is also involved in a

number of other diseases, the use of AEB could be extended potentially to include conditions such as psoriasis and ulcerative colitis.

Preclinical Pharmacology

AEB-071 has been shown to competitively and reversibly inhibit both classical and novel PKC isoforms with an IC_{50} of ~1.5nM in an *in vitro* model using human CD4+ T-cells(7, 8). This inhibition was found to be highly selective sparing other Ser/Thr and Tyr kinases(7). AEB can further inhibit the release of IL-2 from T cells with an IC_{50} of 50nM both in mice(9) and humans(8). More recent reports have shown that AEB inhibits T-cell proliferation induced by CD3/CD28 antibody and alloantigens and prevents LFA-1 mediated T-cell adhesion at nanomolar concentrations(10).

In contrast to cyclosporine (CsA), it is a very weak inhibitor of nuclear factor of activated T cells (NF-AT) with an IC_{50} > 1000nM(8). From a clinical perspective, this may allow AEB to avoid some of the side effects associated with NF-AT blockade, including an alteration in glucose homeostasis(11). Provided AEB can provide similar immunosuppressive effects to CsA and other immunosuppressive agents, it may in fact be a much better tolerated option. In addition, AEB does not have any effect on cytokine or growth factor-induced cell proliferation nor does it modulate T cell apoptosis induced by various stimuli(9). Overall, AEB's mode of

action is distinct from that of the calcineurin inhibitors although it has complementary effects with CsA on T-cell signaling pathways.

Owing to its effects on T cell activation, AEB has been explored in the area of cellular and organ transplantation. Using a rat cardiac allograft model, it was shown that AEB (30mg/kg po bid) prolongs graft survival from 7 days to >28 days(12). Furthermore, non-therapeutic doses of AEB (10mg/kg po bid) combined with low-dose CsA, everolimus or FTY720 resulted in prolonged graft survival to 26, >68 and >68 days respectively(12). A further study in non-human primates showed >100 day renal allograft survival when non-therapeutic doses of AEB were combined with low-dose CsA(8). These initial preclinical studies demonstrate that AEB can delay allograft rejection either as monotherapy or in conjunction with CNIs in a variety of animal models and transplantation settings.

Our group has expanded these preclinical results to the area of pancreatic islet transplantation(13). Currently, islet transplant recipients face a decline in metabolic function probably due to a combination of allograft rejection, type 1 diabetes recurrence and immunosuppressive toxicity. As a result, new more effective and less toxic immune modulating agents have the potential to bring the field to a new era. AEB can delay rat islet allograft rejection when used alone (from 7 to 22 days) or in combination with CsA (>100days vs 12 days for CsA alone)(14). These investigations also demonstrated that AEB has no diabetogenic toxic effects as assessed by glucose tolerance testing and

pancreatic insulin content. Furthermore, AEB placed in culture with human islets for 48 hours has no effect on insulin release nor islet apoptosis(15). Finally, human islets transplanted into an immunodeficient mouse model displayed improved engraftment and sustained function when treated with AEB as compared to sirolimus, a drug commonly used after clinical islet transplantation(15). AEB is therefore of particular interest in the setting of islet transplantation where optimal post-transplant immunosuppression has not been achieved thus far.

AEB has been investigated in the setting of other T-cell mediated diseases including models of type 1 diabetes. Unfortunately, the effect on diabetes onset in the NOD mouse was only moderate; a delay of 2 weeks(16).

Overall, it would seem that AEB has a bright future in the realm of transplantation. It has displayed the ability to prolong graft survival both in solid organ and islet transplantation through the inhibition of PKC; a mechanism wholly different from the CNIs. It has also proved potentially synergistic to the CNIs; a means whereby CNI toxicity could be limited if the two drug classes are combined. Lastly, AEB has proven non-toxic to both native and transplanted pancreatic beta-cells.

Pharmacokinetics and Metabolism

A number of studies have explored the pharmacokinetics and metabolism of AEB in human subjects. Slade et al. used healthy volunteers, administering doses ranging from 25 to 200mg po bid for 14 days. They found that the oral clearance of AEB was time-independent, with steady state being reached by day two (four doses)(17).

An additional study by Skvara et al., administering healthy subjects oral doses from 10 to 500mg, found a dose-dependent inhibition of T-cell proliferation which peaks 1-3 hours after an oral dose(18). This was associated with a concomitant reduction in IL-2 mRNA expression. The biochemical half-life ($t_{1/2}$) of AEB in these healthy subjects was 6 hours. The maximum plasma concentration (C_{max}) increased in a dose-dependent manner, reaching a high of 4μM in the 500mg group (single dose). This study also compared various oral doses to placebo in patients with documented psoriasis(18). Here, the C_{max} reached a high of 4.5μM in the 300mg bid group. Interestingly, the C_{min} in this group was 1.5μM, higher than the IC_{50} for the inhibition of proliferation and IL-2 mRNA expression. Steady state levels were reached after two doses in psoriasis patients.

AEB is known to be highly protein bound and is metabolized by the liver through the Cytochrome P450 system (CYP3A4)(19, 20). This has implications for its use in liver transplantation and in patients with liver failure. Slade et al. showed

that in patients with severe liver impairment (Child-Pugh 10-13) there is a 52% decrease in C_{max}, a 19% decrease in the area under the curve (AUC) and a 2-fold increase in the half-life (12.5 hours) as compared to healthy subjects(19). More importantly, unbound (free) AEB levels were similar between subjects with hepatic impairment and control (normal) subjects. Thus, although there may be lower total levels of AEB in the case of hepatic impairment, the biologically active (free) AEB exposure is not affected.

Overall, AEB appears to reach an effective plasma concentration within a few doses. A twice-daily dosing regimen appears adequate. Although it is metabolized by the liver, exposure to its biologically active form is not altered in hepatic failure. This should allow AEB to be further developed in all types of transplantation, including liver transplantation.

Safety

AEB has been investigated in healthy subjects as well as in patients with psoriasis, liver disease and renal transplants. In a study of 48 healthy volunteers given a single dose of AEB, a total of 12 dose-independent adverse events were observed(18). Self-reported adverse events ranged from mild headache to dizziness. Furthermore, a transient reversible tachycardia was documented in the 500mg bid group lasting from 3 to 12 hours after oral dosing (heart rate never exceeded the upper limit of normal).

In a Phase I safety trial of patients with psoriasis an adverse event ratio of 45.8% in the AEB group was seen (vs 37.5% in the placebo group)(18). These events were mild with nausea predominating in the higher dosing groups. Of note, two out of 32 patients experienced elevations in alanine aminotransferase (ALT). In both patients these levels returned to normal with continued AEB treatment. Renal function was reported as normal throughout this study.

Recently, two trials utilizing AEB in renal transplant recipients have been reported(21, 22). The first compared AEB (200mg bid) plus tacrolimus (Tac) (with Tac conversion to mycophenolate at 3 months) to tacrolimus plus mycophenolate (MPA)(21). Safety was comparable between the two groups with similar rates of drug discontinuation due to adverse events and similar rates of infections. Notably, the AEB group displayed a lower incidence of neutropenia (3-4%) when compared to Tac/MPA(11%). The second study compared AEB (300mg bid) directly to Tac(22). There was a higher incidence of gastrointestinal (GI) adverse events (nausea, vomiting or constipation) on AEB although the incidence of diarrhea was similar. As in the healthy subjects discussed above(18), heart rate was 2-10bpm higher in the AEB group with a higher incidence of tachycardia (increase of >25% from baseline, >100bpm). Patients on AEB obtained a benefit in renal function as compared to the Tac group (GFR of 70ml/min vs 48ml/min).

Calcineurin inhibitors have long been known for their potent immunosuppressive ability, however, they suffer from numerous side-effects and toxicities. AEB appears to have acceptable tolerability with some benefit to renal function and neutrophil count. Further studies are still needed to optimize the dosing of AEB but, in terms of toxicity, it appears to be a potentially useful adjunct to the CNIs.

Clinical Studies

Nine unique clinical trials employing AEB are currently registered under the National Institutes of Health registry ClinicalTrials.gov (Table A2-1). These include a mixture of dose-finding trials (n=4), trials of efficacy in solid organ transplantation (n=3) and other immune-mediated processes (n=2). To date, the only data published in a peer-reviewed journal is that of the psoriasis study which showed a dose-dependent improvement in psoriasis severity during the treatment period(18). The most remarkable clinical improvement was seen in the 300mg bid group (the highest dose employed) where they showed a 69% reduction in psoriasis severity index. The drug was discontinued after two weeks and all groups except the 300mg group returned to baseline severity after this point. Histological changes, more specifically a significant reduction in T cells, were observed in this group.

A recent review published by Vincenti and Kirk shed some light on the preliminary results of three of the ongoing clinical trials(23). The first study used a

regimen of AEB & Tac with Tac withdrawal at three months compared to Tac and mycophenolate (MPA) in the setting of renal transplantation(21). While efficacy failure was similar at 3 months, the study was terminated prematurely. This occurred in the absence of Tac (after withdrawal) due to a significant increase in efficacy failure (defined as biopsy proven acute rejection, graft loss, death or loss to follow-up). In a second study, AEB was compared directly to Tacrolimus(22). This study was also prematurely halted due to an increase in rejection in the AEB group (26% vs 5% at 3 months). A third trial is ongoing comparing AEB used in combination with everolimus and steroids to Tac with MPA and steroids.

AEB has already shown promise as an adjunct in organ transplantation and as a powerful treatment for psoriasis. In the renal transplant studies, AEB was paired with mycophenolate. Our group has shown that this combination is ineffective in preventing rejection in islet transplantation in rats. A more fruitful combination may be AEB paired with low dose CsA; a combination we showed to prolong graft rejection >100 days. In fact, Budde et al. showed comparable efficacy failure between AEB paired with low-dose Tac and Tac plus mycophenolate(21). It also remains to be seen whether the above preclinical studies in islet transplantation can be applied in a clinical setting.

The most recent updates presented at the 2009 American Transplant Congress clearly suggest that AEB must be combined with a CNI (Tac or Neoral) in order to maximize protection against acute rejection events(21). AEB + mycophenolate

mofetil (MMF) was unable to provide adequate prophylaxis against acute rejection in the absence of CNIs(22). An ongoing Phase II study of AEB + everolimus (a rapamycin derivative) is underway in renal transplantation, and results are eagerly awaited. It seems most likely that AEB will need to be combined with half-dose Tac or Neoral to find its appropriate niche in the transplantation armamentarium.

Drug Interactions

AEB has been shown to work synergistically at non-therapeutic doses with cyclosporine, everolimus and FTY720 in various transplantation models(8, 12, 14, 20). The delayed rejection observed in rodent models is not thought to be as a result of pharmacokinetic interactions between these drugs(12). Even so, Slade et al. showed that when AEB and CsA are combined, there is a dose-dependent increase in the AUC of AEB(20). In healthy human subjects, AEB and CsA work synergistically to provide a significant reduction in markers of T-cell activation and an additive inhibitory effect on lymphocyte proliferation similar to high-dose CsA alone(20). Additionally, AEB works synergistically with mycophenolate to provide an increase in anti-proliferative activity (~50% individually vs 80% in combination)(17).

It is still likely that the significant effects of AEB combined with CsA are due to synergism through their closely related pathways. This evidence should provide

further support for the development of AEB/low dose CsA based immunosuppressive regimens.

Conclusion

Organ transplantation continues to be a life-saving procedure performed across the globe. New immunosuppressive agents are constantly being sought in order to provide safer and more effective means of preventing graft rejection. In pre-clinical studies AEB, a protein kinase C inhibitor, has proven to be a viable alternative to the calcineurin inhibitors, a class of drugs which have proven effective yet linked to numerous side effects. While early clinical trials have proven AEB safe, they have failed to show any ability for AEB to be effective as a monotherapy. This does not mean that AEB should be ignored; it may yet find as role in combination with other agents, especially if those agents are given at doses which reduce their potential side effects.

Figure A2-1 Mechanism of action of AEB-071

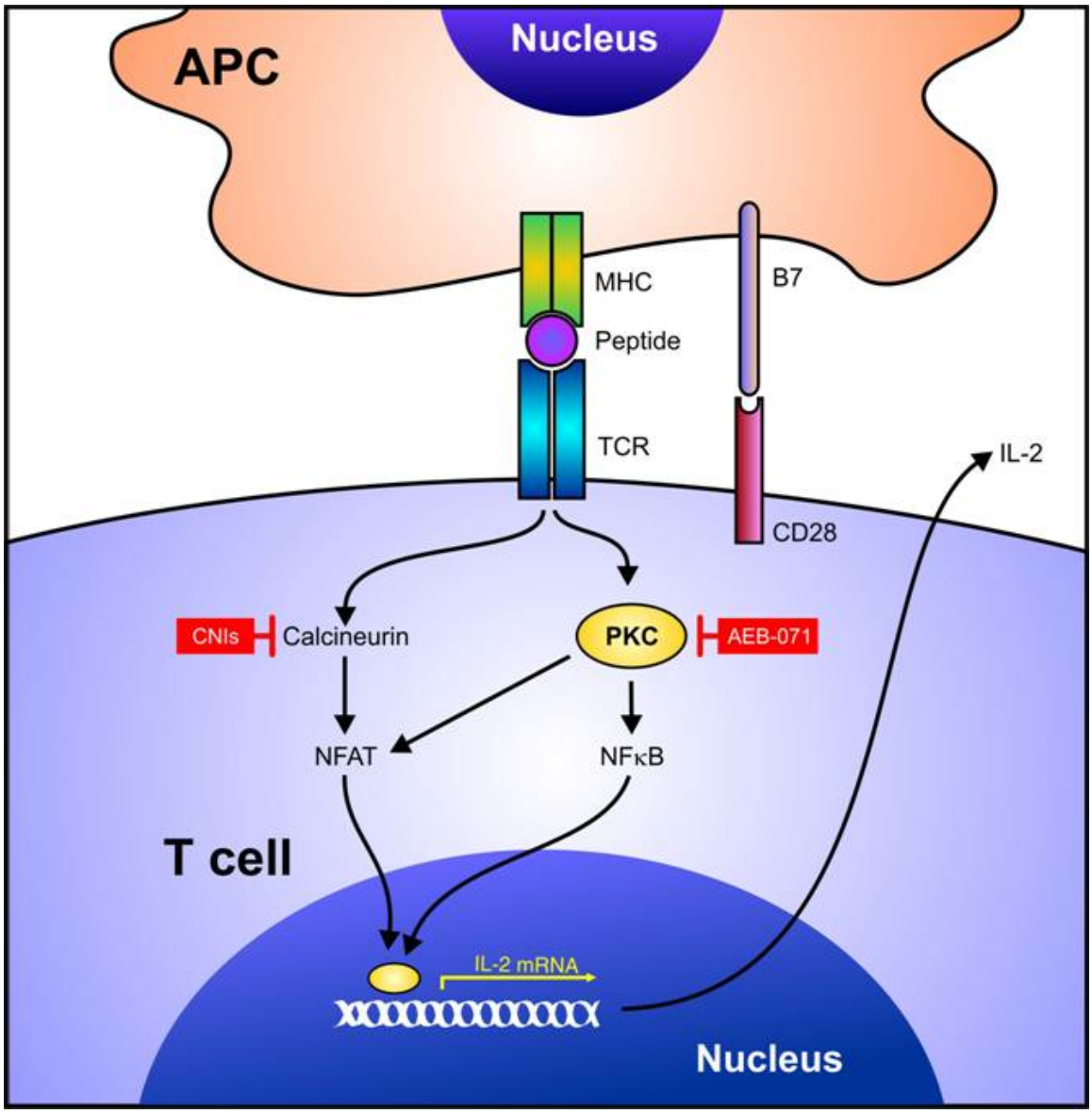

Table A2-1 Current and completed clinical trials involving AEB-071

Condition	Purpose of study	Primary outcome	Treatment group	Comparison group	Phase	Status
Healthy	Safety; Caucasians vs Japanese subjects	PK, safety, tolerability	AEB	Placebo	Phase I	Completed
Healthy	Safety and PK of doses >500mg	Safety, tolerability, maximum tolerated dose	AEB	Placebo	Phase I	Completed
Renal Transplantation	Efficacy	Acute rejection, graft loss	AEB + everolimus + basiliximab + CS	Cyclosporine+ everolimus + basiliximab + CS	Phase II	Recruiting
Renal Transplantation	Efficacy and safety	Long-term safety >12 months(renal)	AEB + Tac with conversion to AEB and MPA at 3 months	Tac + MPA	Phase II	Completed
Renal Transplantation	Efficacy and safety	Acute rejection, graft loss	AEB + Basiliximab + MA + CS	Tac + MPA + Basiliximab + CS	Phase II	Completed
Liver Transplantation	PK	PK, safety, tolerability after single dose	AEB	N/A	Phase I	Completed
Psoriasis	Dose finding using 20-200mg bid doses	Change in plaque severity	AEB	Placebo	Phase II	Recruiting
Ulcerative Colitis	Efficacy and safety	Rate of remission induction	AEB	Placebo	Phase II	Suspended
Uveitis	Safety, tolerability, efficacy	Safety and tolerability	AEB	N/A	Phase II	Recruiting

AEB = AEB-071 (sotrastaurin), CS = corticosteroids, MPA = mycophenolic acid, PK = pharmacokinetics, Tac = tacrolimus,

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