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Beta Cell Replacement Therapy

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1. Introduction

Type 1 Diabetes mellitus (T1D) is an autoimmune disorder caused by destruction of pancreatic beta cells which produce insulin. Current estimates have shown that T1D affects about 0.4 to 0.8% of the population worldwide accounting for 5-10% of all diabetes cases (Stock and Bluestone, 2004). It is seen as an increasing health hazard. For T1D, exogenous insulin administration is the most widely used treatment. However, this treatment has several limitations including development of secondary health complications over time. There are several approaches already in practice to replace the damaged beta cells in T1D. They include whole organ pancreas or isolated islet cell transplantation. Several novel approaches are currently in development such as expansion of adult beta cells and stem/progenitor cells which can be transformed into beta cells. This review summarizes recent progress made in beta cell replacement therapy for T1D. Diabetes mellitus has been known for thousands of years. Diabetes comes from an ancient Greek word coined by Arataeus of Cappadocia, and mellitus from the Latin word honey associated with sweet urine. (Medvei, 1993).

The pathogenesis of T1D has been attributed to an autoimmune response. Currently the mechanism that triggers this disease is still unknown, but it seems to be a combination of environmental and genetic factors. T-cells in the host's body become sensitized to protein that naturally occurs in the beta cells of the pancreas and the immune system begins to mount a specific attack leading to destruction of the islets of Langerhans. Several candidate proteins have been identified including GAD65, insulin, and ZnT8 (Harlan et al., 2009, Shapiro et al., 2003). Reducing the autoimmune effect in beta cell replacement is very important, but before moving onto the strategies for beta cell replacement therapy it is important to describe the standard therapy for T1D and how it falls short of providing for all the needs of diabetic patients.

Exogenous insulin therapy continues to be the leading therapy for T1D today, but there are several complications associated with it. However, even intensive monitoring of blood glucose and injection of insulin is not enough to halt the secondary complications of diabetes (Harlan et al., 2009). Diabetes mellitus can decrease patients' lifespan, especially in severe cases. Lowered blood sugar levels, which can result if too much insulin is administered, can also lead to a hypoglycemic episode (Cooke and Plotnick, 2008). These episodes are typified by ketoacidosis, where in an effort to find energy, the body burns fatty acids leading to the production of acidic ketones that can be detected by an alcoholic smell on the breath. Hypoglycemic episodes can lead to coma and even death, and is considered

the most severe side effect of diabetes. Secondary complications such as blindness, peripheral neuropathy, and cardiovascular complications result from destruction of microvasculature. Additionally, the quality of life is lower even for patients with well regulated blood sugar.

There are several methods both clinical and experimental that are being developed to provide optimal beta cell replacement. The first type that was developed and successfully applied to the clinical setting was that of whole organ pancreas transplant (WOP). Next, in an effort to transplant only the insulin secreting portion of the pancreas, islet transplantation was explored and has been applied through several clinical trials. Efforts have been made to improve this technique by perfecting the techniques of islet isolation and immunosuppression; immunoisolation and xenotransplantation are also being explored. However, even if all donor human pancreases were effectively used, they would be insufficient to treat every patient eligible for transplant. This emphasizes the importance of current studies into beta cell regeneration from many sources including embryonic stem cells, and pancreatic ductal and acinar cells.

2. Pancreas transplantation

Transplantation of whole allogenic pancreas is an established procedure. The first clinical transplant of a whole vascularized pancreas was at the University of Minnesota in 1966. Initial results showed very poor graft survival rates; less than 5% of grafts survived after six months. Important advances were made in surgical technique and post-transplant monitoring. Because the pancreas is a very large, immunogenic organ, large doses of immunosuppressants are required to prevent rejection. Immunosuppression protocols had to be adjusted to prevent graft rejection while not being so strong as to damage the beta cells. However, the addition of mycophenolate mofetil and tacrolimus to regimens suppressing the immune system dramatically increased the success of whole pancreas transplant from 20% to 80% survival after one year. One important stepping stone in anti-rejection therapy was the elimination of steroids. The addition of sirolimus, an immunosuppressant lacking nephrotoxic or beta cell toxic properties, along with an antilymphocyte antibody induction allowed the discontinuation of steroids from maintenance therapy. This immune therapy led to a low rejection rate of pancreas. Three year survival rate, defined as insulin independence, is around 80% according to the International Pancreas Transplant Registry (Gruessner, 2001).

Transplanting a whole pancreas simultaneously or after a kidney transplant has provided significant improvement in patients. Since diabetes affects the function of the renal system, many patients will have a history of dialysis. Such an issue would call for a kidney transplant, and since this would already require a surgical procedure and immunosuppression, adding a pancreas transplant in a simultaneous fashion could prove beneficial (Humar et al., 2000). In fact, the development of secondary complications is attenuated by an allogenic pancreas transplant. Further, the progression of nephropathy is decreased from diabetic effects with the simultaneous transplant of a pancreas and kidney. There also appears to be improvements to the quality of life and in the damage to the peripheral nerve system (Venstrom et al., 2003).

Although transplant of whole allogenic pancreases offers the above advantages, it comes with the serious complications associated with surgery. A study by the University of Pennsylvania (Frank et al., 2004) compared 30 whole pancreas and 27 isolated islet

transplants. This investigation showed that 7 patients (23%) required post-transplant surgery while only 1 islet recipient (7%) needed surgery. There was a significant difference in the number of patients requiring blood transfusions post-transplant from whole pancreas (43%) versus islet transplant (7%). The main reason that would account for the differences in surgical complications can be attributed to the invasiveness of the whole pancreas transplant versus the relatively non-invasive islet transplant, which is performed by percutaneous puncture and infusion into the portal vein of the liver. Thus long hospital stays are required of whole pancreas transplant cases whereas islet transplantation is an outpatient procedure leading to a lower estimated cost. Despite the lower surgical risk and cost of islet transplantation it has not yet achieved the duration or rate of graft success found in whole pancreas transplantation.

An issue for beta cell replacement is the low number of donors compared to the number of eligible recipients (Matsumoto et al., 2006). In the United States, there are an estimated 7,000 available donor pancreases, but the number of pancreases available for clinical transplant are much lower due to many exclusion criteria. For whole organ transplant, young donors (<50 years old), with low BMI are preferred. Although the islets received from young donors are suggested to display greater functionality, it is very difficult to effectively isolate a sufficient number of islets for transplant from these donors. Also, obese donor pancreases are associated with higher surgical complication rates. Conversely, isolations from pancreata from older, obese (BMI>30) donors produce higher yields of islets that are still functional and qualify for clinical transplant. Thus islet transplant has the ability to utilize marginally acceptable pancreases for clinical transplant, increasing the ability to perform beta cell replacement. These non-overlapping criteria for whole organ and islet isolation reinforce the notion that these two treatments are not competitive, but rather complementary.

The important advances and improvements of whole organ pancreas transplant over the past two decades have given significant momentum to this method of beta cell replacement in patients with life-threatening diabetes. Although the one year rejection rate is higher at 8% for solitary pancreas transplant in preuremic patients than the rejection rate of 2% seen in persons receiving simultaneous pancreas and kidney transplants, the increased success of the procedure still warrants its application (Gruessner, 2001). However, this treatment is still limited to patients with brittle diabetes experiencing uncontrolled blood glucose levels and hypoglycemic unawareness despite intensive insulin therapy. The risk of morbidity, the need to open the abdominal cavity during surgery, and the strong immunosuppressive regimen required for solid organ transplant substantiates a long, hard look at alternative methods of beta cell replacement.

3. Islet cell transplantation

The transplantation of pancreatic islets of Langerhans is an exciting alternative, because it reduces the surgical risk of complication by being less invasive (Frank et al., 2004). Furthermore, transplanting only the functional, insulin secreting portion of the pancreas reduces the risk of activating the exocrine, digestive portion of the pancreas, which could lead to deterioration of endocrine function.

Islet transplantation also provides several other exciting possibilities. One such possibility is the ability to manipulate islets prior to implantation to protect them from the immune system or to attach biologically active molecules that could aid in engraftment. Beta cell regeneration by embryonic or adult stem cells also provides the possibility of a large

population of renewable insulin secreting cells, but the risk of neoplasm formation still remains a potential issue (Borowiak & Melton, 2009; Ricordi & Edlund, 2008). Xenogenic, porcine islets could also fill the gap between beta cell supply and demand (Hering & Walawalkar, 2009). Each of these methods contains complicating factors that do not allow current application to the clinical setting, but recent advances have put them closer in reach than ever before.

A comparison of the historical progress of whole organ transplant to that of islet transplantation would provide an objective basis to evaluate the progress of islet transplantation. Although whole organ treatment achieved high levels of graft survival in the years 1994-1997, the islet survival rate at five years has reached around fifty percent in 2010-2011, comparable to the level of whole pancreas graft success (Rickels et al, 2011). From this perspective, islet transplantation is not inferior to pancreas organ transplantation, though it may not have reached its full potential yet.

In the past decade since the publication of the Edmonton Protocol (Shapiro et al., 2000), there have been many advances in the field of islet transplantation, primarily in the area of islet isolation technique and immunosuppression therapy. Breakthroughs have been made in the area of pancreas procurement and preservation with study into ductal preservation, the two layer method, and the type of preservation solution used. Furthermore, there has been much progress in the islet isolation process by bringing standards up to cGMP qualifications, optimization of collagenase enzymes, and using iodixanol for continuous density gradient purification (Matsumoto et al., 2009). With the introduction of thymoglobulin at induction phase and the combination of prograf and mycophenolate mofetil as maintenance immunosuppressive agents, the islet transplant survival rate has significantly improved recently.

3.1 Pancreas preservation and islet isolation

The islet isolation process is fundamental to islet transplantation. The main stages in isolation are pancreas procurement and preservation, digestion of the organ, and purification of the islets. First, the pancreas is obtained from the organ procurement organization, prepared with certain preservation solutions and injections as described later. Upon arrival at the isolation facility, the pancreas is disinfected and injected with a digestion enzyme that degrades the pancreas and releases islets. Lastly, the mixture of pancreatic tissue is centrifuged with a density gradient to separate the endocrine tissue from the exocrine tissue. Within each of these procedures are detailed methodologies that have been explored to improve both islet yield and function.

3.1.1 cGMP facility

One major requirement for islet cell preparations and isolations is that it should be performed under current good manufacturing practices (cGMP). An important requirement of cGMP facilities is the validation and sterilization of all equipment (Garfinkel et al., 2004). Although initially assumed to increase the cost of establishing an islet isolation program, the guidelines of cGMP ensure the efforts of improving safety of islet preparations. Documentation of errors, corrective measures, and preventative actions minimize costs and provides the ability to critically evaluate the impact of certain actions. Validation of instruments prevents false bias readings, and validation of processes indicates whether assumptions about the production are correct or not (Yamamoto et al., 2009).

Documentation not only proves the correctness of the manufacturing, but also provides data for scientific reports. However, some of these requirements still can create a large amount of work.

3.1.2 Pancreas preservation

In the area of organ preservation, damage due to low oxygenation and warm ischemia are important hurdles to overcome. Warm ischemia is the time between the cessation of a heart beat in a donor's body and when cold preservation solutions can be injected. Ischemia is the loss of blood circulation to an organ that consequently causes a lack of oxygen, glucose, and other helpful molecules in the blood. In general, warm ischemia times greater than fifteen minutes cause pancreases to be of marginal quality and times exceeding thirty minutes usually disqualifies the organ for transplantation. Significant amounts of damage are incurred to the pancreas and islets during warm ischemia time, but cold ischemia time should also be limited as much as possible. Cold ischemia time is incurred during procurement and transportation of the organ, and should be lower than twelve hours. In addition to ischemic injury, there is also the danger of hypoxic damage from low oxygenation conditions (Sawada et al., 2003). Following procurement, donor pancreases were initially preserved in University of Wisconsin solution (UW) or Histidine-Tryptophan-Ketoglutarate solution (HTK). Although these solutions are standard for tissue preservation of other organs, other preservation methods were attempted to improve the results of islet isolation. The two layer method and ductal preservation have emerged as two techniques that preserve the pancreas while leading to increased islet yield and quality from isolation.

The advent of perfluorocarbon as an excellent oxygen carrier led to significant improvements in reducing hypoxic damage. Perfluorocarbon (PFC) is a hydrocarbon derived chemical where all of the hydrogens of a carbon chain are replaced by fluorine atoms. The uniform covering of fluorines prevents polarization of the electron clouds, which explains the low van der Waals forces and non-polar nature of the molecule (Lamal, 2004). The property of PFC that makes it particularly attractive as an organ preservation and oxygenation solution is the capacity to dissolve large amounts of oxygen. This high dissolving power of PFC combined with a low oxygen binding constant, due to low intermolecular forces, allows PFC to release oxygen more effectively than hemoglobin (F2 Chemicals Ltd., 2003).

The Two-Layer Method (TLM) involves placing PFC, which is almost twice as dense as water, beneath the UW/HTK solution and oxygenating the solution before adding a procured pancreas. The addition of a water based preservation solution above the PFC solution prevented the loss of dissolved oxygen (Matsumoto & Kuroda, 2002). The distinction between the static and original TLM is that the original method provides continuous oxygen supplied to the PFC, while static method precharges the PFC with oxygen before the organ is added. Cold ischemia time for human pancreata can be extended up to thirteen hours using TLM. Islet yield, viability, and functionality were significantly improved by both two-layer methods compared to UW solution alone. The human islet isolations with the two layer methods yielded about twice as many islets ($2,659 \pm 549$ IEQ/g in the static TLM, $2,244 \pm 557$ IEQ/g in the original TLM) compared to the UW method ($1,293 \pm 451$ IEQ/g). This study showed that the islet yield, viability, and *in-vitro* function were significantly improved by both TLM approaches with similar results. Since the static

TLM is easier and yields comparable results as the original method it has subsequently attained widespread use in clinical studies. In contrast, a larger study comparing the effectiveness of TLM vs preservation in UW solution alone reported no significant advantage in using TLM method (Caballero-Corbalan et al., 2007)

Although this TLM is effective at oxygenating the pancreas, the organ is still a thick tissue with inner, non-accessed parts being exposed to a higher risk of damage. It has been hypothesized that the ductal system is thus especially susceptible to cold ischemic injury. This could result in the inability to properly perfuse collagenase (an essential reagent discussed later) by intraductal distention. By injecting cold preservation solution in the opposite direction through the main pancreatic duct, a majority of the inner spaces of the pancreas can be reached, preserving a greater number of islets for isolation, and allowing greater distention of the organ during collagenase injection (Matsumoto et al., 2002). Functionality results for islets isolated from pancreata receiving ductal injections of cold preservation solution were evaluated by an *in-vivo* assay, insulin secretion, and by viability testing. Ductal preservation significantly reduced the number of nonviable cells from around sixty percent to lower than twenty percent. Using this method, the insulin secretion ability of islets was also improved. The best determinant of islet quality is the *in-vivo* assay, which involves the transplantation of isolated islets under the kidney capsule of a diabetic nude mouse. This further confirmed that islets not preserved with ductal injection could not cure diabetic mice in any of the cases, whereas islets from pancreata perfused with UW solution showed a similar curative rate compared to islets from a fresh pancreas (Sawada et al., 2003).

Protecting the pancreas from ischemic damage between procurement and processing is an important aspect of islet isolation. Although islets only constitute about 2-5% of the total pancreatic mass, they use over 10% of the organ's blood supply. Therefore, sufficient oxygenation is important for islet function. Previous studies performed have demonstrated the ability of the two layer method and ductal preservation to yield higher numbers of improved quality islets.

3.1.3 Collagenase

Pancreas digestion is performed by injection of collagenase enzyme that cleaves the basic connective tissue. Successful human islet isolations hinge upon the quality of the collagenase used, which is the blade that cuts islets from the pancreas providing isolated islets. The types of collagenase traditionally used are derived from *Clostridium histolyticum* (Linetsky et al., 1997). Liberase HI is a blend of purified collagenases and protease that showed significantly higher islet yields when compared to crude collagenase extracts (Linetsky et al., 1997, Olack et al., 1999). Liberase HI provided the advantage of not only higher islet yields compared to crude collagenase, but also a reduction in contaminating enzymes and endotoxin. Despite these advantages, Liberase HI appeared to have inconsistencies between lots, and exhibited poor storage stability (Barnett et al., 2005). This enzyme blend further fell out of favor, because animal products such as bovine brain extract were used in the manufacturing process (Shimoda et al., 2010). Many centers turned to NB-1 collagenase products (SERVA/Nordmark) in order to avoid the potential safety risks (Bucher et al., 2005, Shimoda et al., 2010). Results from many centers that used this enzyme are mixed in terms of generating islet yields and quality similar to those achieved by Liberase HI (Barnett et al., 2005; Yamamoto, 2007).

To show the further usefulness of an optimal collagenase, human pancreas isolations were performed by Balamurugan et al., (2010) with collagenase from VitaCyte (n=28) and Serva/Nordmark (n=30). The results of these isolations show that VIzyme produced significantly higher yields of islets. The final islet equivalent (IEQ) per gram of pancreas for VitaCyte enzyme versus Serva's enzyme was $414,700 \pm 175,900$ versus $213,400 \pm 152,400$ with a p value of 0.002. Islet equivalent (IEQ) is the standard unit of measurement of islets and corresponds to a round islet with a diameter of 150 micrometers. From these isolation results, six clinical islet transplantations were performed. Two patients received islets isolated from Serva's NB-1; one patient achieved normoglycemia for over 700 days and the other had evidence of graft failure on day 84. Of four patients transplanted with islets isolated using collagenase from VitaCyte, three were insulin independent for more than 350 days. Although this clinical data is not conclusive, it does demonstrate that the utilization of VIzyme in islet isolation may prove beneficial to increasing islet yields. Further independent studies are warranted to validate such claims.

3.1.4 Purification

The final stage in the islet isolation process is purification to separate functional islets, from the contaminating acinar cells. The positive aspect of this process is a higher purity of islets to transplant; also the packed cell volume will be lower, decreasing the risk of an embolism or other risks associated with the islet infusion process.

This separation process takes advantage of the different densities of acinar and islet cells, with acinar tissue being more dense than islets. However, one disadvantage is that the islet post-purification recovery is mostly decreased. The traditional gold standard for this process was the use of Ficoll, a heavy, multi-branched sugar that readily dissolves in water, based density gradient combined with semiautomated centrifugation with a COBE-2991 cell processor to achieve optimal results (Matsumoto et al., 2006). The centrifugation process can be mechanically stressful and damaging to islets; further the exposure to enzyme and endotoxin in the isolation can lead to apoptosis, inflammation, and attack by the immune system post-transplant. An alternative density solution, iodixanol, is a neutral iso-osmotic contrast solution used clinically in the imaging field. Iodixanol's effect on the purification was studied along with the production of pro-inflammatory cytokines after islet isolation (Mita et al., 2010). Islets purified by iodixanol-based gradients yielded significantly lower levels of cytokines and chemokines when compared to Ficoll-based gradient solutions. The inflammatory molecules that were downregulated include interleukin-1 beta (IL-1 β), tumor necrosis factor-alpha (TNF- α), interferon-gamma (IFN- γ), IL-6, IL-8, macrophage inflammatory protein 1beta (MIP-1 β), and monocyte chemoattractant-1 (MCP-1). These cytokines are known to not only be pro-inflammatory, but some are also associated with apoptosis and necrosis. These results provide compelling data that iodixanol has a protective effect on islet preparations when used in density gradient purifications. Furthermore, Hering et al. (2005) have reported that islet preparations purified using iodixanol-based gradients were able to cure eight patients with a single transplantation.

In the Edmonton protocol (2000), islets were transplanted immediately after isolation, without a period of culturing. Although this may cause attrition of islets, some argue that the culture period allows damaged ineffective islets to die off while permitting enough time for functional testing of islets prior to transplantation. Further, the culturing period could be used to pretreat the recipient or the islets themselves.

The culture of islets up to 48 hours pre-transplant has gained some popularity for several reasons. This culture time allows depletion of apoptotic islets and testing for sterility, microbiology, viability, and *in vitro* glucose stimulated insulin secretion response. The patient does not have to be rushed into a radiology suite to receive the infusion, but this time could allow for the start of an immune cell depleting induction therapy.

Thus, the advances made in the area of islet isolation have been substantial over the past decade. The standardization to cGMP facilities, preservation with the two layer method and ductal injection method have all improved the preparative conditions for conducting an isolation. The optimization of the collagenase solution continues to improve islet yields after pancreatic digestion. By currently using iodixanol based density gradient solutions, the risk of apoptotic islet damage both after isolation and after transplantation has been reduced by the lowering of inflammatory cytokines. These improvements have led to higher yields and islets with greater functionality, which provides transplant recipients a greater chance at achieving insulin independence.

3.2 Advances in immunosuppression to prevent islet graft rejection

Although it is difficult to obtain islet preparations of an adequate quantity and quality for transplantation, it is also very difficult to protect and maintain the allogenic islet function after transplantation. There are several aspects of the immune system that prevent the long term survival of islet cells. The first reaction encountered by islets is the instant blood mediated inflammatory response (IBMIR), primarily mediated by the innate immune system which leads to islet destruction (Bennett et al., 1999). Islets surviving this initial, short term inflammatory response are later subjected to targeting of autoimmune and alloimmune responses. The alloimmune response is primarily directed against mismatched HLA. The autoimmune response which already exists in type 1 diabetic recipients, being the source of the pathogenesis of the disease and is specifically reactive to beta cell markers such as glutamic acid decarboxylase (GAD65) and islet cell antigen (ICA) (Shapiro et al., 2003). Immunosuppressive regimens for islet cell transplant recipients must counter these aspects of the whole immune response for improved graft survival.

Immunosuppression after islet transplantation is comprised of two phases namely induction and maintenance therapies. The induction phase is characterized by anti-inflammatory drugs and monoclonal or polyclonal antibodies that deplete immune cells (anti-CD3 and anti-thymocyte globulin) or prevent T-cell activation (anti-IL-2 receptor) (Stock & Bluestone, 2004; Matsumoto et al., 2011). The maintenance drug regimen is focused on suppression of T-cells by various strategies including calcineurin inhibitors such as tacrolimus, and cyclosporine. Other drugs such as mycophenolate mofetil (MMF) have also been used with good outcomes.

In the early days of islet transplantation, drugs administered to counter the immune system were found to be toxic to islet cells (Stock & Bluestone, 2004). Beta cells naturally have lower levels of anti-oxidant enzymes, which put them at an increased risk. Further, the hepatic graft site puts islets in contact with blood that has higher concentrations of the immunosuppressive drugs, increasing any negative side effects. The advent of the Edmonton protocol demonstrated successful islet transplantations, with a part of the accomplishment being attributed to minimizing calcineurin inhibitors and a steroid-free induction process.

3.2.1 Induction therapy

The first stage of treatment for islet transplantation is the induction therapy that attempts to reduce the inflammatory effects of the short term immune system. It has been estimated that during the initial phase after transplant, as much as 50 to 60% of the islets graft is lost due to IBMIR (Bennett, 1999). Within as little as five minutes, natural IgG and IgM lead to complement activation starts to attack it by creating perforin complexes that lead to cell lysis (Tiernberg, 2008). Another significant physiological change involved in IBMIR is the dramatic, rapid increase in cytokine levels such as IL-2, TNF- α , IL-1 β , and IFN- γ . Due to diabetogenic effects glucocorticoids are avoided in the immunosuppressive regimens turning to newer alternatives (Shapiro et al., 2000). An alternative to steroids is daclizumab, a humanized monoclonal antibody with specific affinity to the IL-2 receptor. This receptor on T-cells is responsible for the main activation pathway, which triggers an immune response against beta cells.

Thymoglobulin has become a popular agent for the reduction in host T-cells (Finke, 2009). This thymoglobulin is a polyclonal antibody that targets T-cells, thus depleting the cells required to mount a specific response to islets even before they enter the body.

Another T-cell depleting agent used for induction therapy is alemtuzumab (Maggiocca, 2006). Alemtuzumab is a monoclonal antibody against CD52, which is a marker of mature lymphocytes. One specific complication is that it increases the chance of infections while also increasing the possibility of reactivation of cytomegalovirus.

Another target of induction therapy includes the proinflammatory cytokine response. Our group and others have shown that IL-1 β plays an important role in the onset of T1D. Additionally TNF- α blockage was shown to significantly improve the clinical outcome of patients based on the collaborative islet transplant registry (CITR) database (CITR Report 2009). To abrogate the inflammatory effects of these cytokines a combined treatment of etanercept and anakinra was used for induction therapy (Matsumoto, 2011). Etanercept is a TNF- α inhibitor, acting as a decoy receptor and blocking the true effect of TNF. Anakinra is an IL-1 receptor antagonist blocking the activity of IL-1 β . This anti-inflammatory response was combined with thymoglobulin for the induction therapy. All patients undergoing this therapy were able to achieve insulin independence following a single islet infusion. Gene microarray analysis was performed on blood samples taken within the first week post-transplant. The results of the gene microarray demonstrated that genes related to cytotoxic T cells were repressed; however, there was upregulation seen in inflammation and neutrophil related genes. This suggests that there may be alternative inflammatory cytokines and agents that may be relevant to the immediate immune reaction.

3.2.2 Inhibition of NF- κ B to prevent inflammation

Instead of trying to target all of the extracellular agents that might cause an inflammatory reaction, an effort was made to find and inhibit a downstream signaling protein that is activated by pro-inflammatory cytokines. Nuclear factor kappa-light-chain-enhancer of activated B-cells (NF- κ B) is a protein that regulates transcription of several sequences of DNA and is activated through a canonical pathway by inflammatory cytokines (Hawiger, 2001). This led us to believe that inhibition of NF- κ B would inhibit the collective effects of circulating cytokines.

An inhibitor of NF- κ B activation was found in traditional eastern medicine from the plant *Withania somnifera* extract called Withaferin A, WA (Kaileh et al., 2007). Withaferin A is a

steroidal lactone that prevents the phosphorylation and subsequent degradation of inhibitor of kappaB (I κ B), which sequesters NF- κ B in the cytoplasm. Studies by Peng et al. (2010) showed that RNA transcripts of inflammatory cytokines and chemokines such as IP-10 (CXCL10), RANTES (CCL5), MIP-1 (CCL4), and MCP-1 (CCL-2) were upregulated in human islets in the presence of cytokines. However, WA was able to inhibit this effect. WA was not shown to decrease islet viability or functionality, while simultaneously inhibiting the inflammatory response. This result demonstrates that careful regulation of NF- κ B can have a beneficial effect for islet transplantation by preventing the effects of cytokine induced damage.

Another NF- κ B inhibitor was found to improve intraportal islet transplantation by IKK- β inhibition (Chen et al., 2011). This study showed higher retention of insulin independence in a xenogenic model when a proprietary NF- κ B inhibitor drug was injected once, thirty minutes before the transplantation. The primary anticipated effect of NF- κ B inhibition is on the IMBIR reaction which is more easily studied in a syngenic model, where the only source of graft failure is IBMIR rather than a specific adaptive immune response.

3.2.3 Maintenance therapy

The purpose of the maintenance therapy is to suppress the specific alloimmune and autoimmune responses mediated by T and B lymphocytes. The drugs typically used for this purpose include Prograf, Rapamycin, MMF, and blockers of co-stimulation. Several combinations of these therapeutic agents have been attempted in clinical islet transplantations to increase the longevity of the graft while minimizing the beta cell toxicity and patient related adverse events.

The types of immunosuppression traditionally used for transplantation are the calcineurin inhibitors tacrolimus and cyclosporine, but often these agents were associated with toxic effects for beta cells (Drachenberg, 1999). Tacrolimus is an immunosuppressant that was found to inhibit NF-AT activation. The original molecule was found in the fermentation of a soil sample containing the bacterium *Streptomyces tsukobensis* (Pritchard, 2005). Tacrolimus inhibits the activation of NF-AT and its translocation to the nucleus by forming a complex with the immunophilin FKBP12 to inhibit the activation of calcineurin and subsequent T-cell activation (Liu, 1991). This action also leads to the arrest of the cell cycle, preventing it from moving from the G₀ stage to the G₁ stage; thus cellular proliferation is prevented. Cyclosporin acts in a similar way as tacrolimus, binding to cyclophilin to prevent calcineurin mediated dephosphorylation of NF-AT; however tacrolimus exhibits greater potency with fewer harmful side effects compared to cyclosporine.

Although the calcineurin inhibitors have been effective at inhibiting rejection in solid organ transplant, they have also been associated with the onset of diabetes mellitus. This is a serious implication for a transplantation procedure trying to prevent this very condition. This side effect was histologically analyzed in solid pancreas transplants for patients receiving either tacrolimus or cyclosporine in a randomized fashion (Drachenberg, 1999). Sectioned biopsy samples from 1 to 8 months post-transplant revealed cytoplasmic swelling, vacuolization, apoptosis and irregular insulin staining of islets for patients receiving both drugs. Although islet cell damage was more frequent for patients receiving tacrolimus versus cyclosporine (10/13 versus 5/13 respectively), it was not significantly different. Since tacrolimus is more effective with less side effects, it is used more often. But as this study shows it should be administered in low doses to prevent beta cell toxicity.

Reference	# of Patients	Insulin Independence (%)	Long Term Follow Up	Immunosuppression
Shapiro et al., 2000	8	100%	18% at 5yrs	Daclizumab (Dac), Rapamycin (Rap), Tacrolimus (Tac)
Noguchi et al., 2005	5	60%	NA	Basiliximab, Rap, Tac
Ryan et al., 2005	65	90%	70% at 1yr 10% at 5yrs	Dac, Rap, Tac
Toso et al., 2006	8	100%	71% at 1 & 2 yrs	Dac, Rap, Tac
Vantyghem et al., 2009	5	60%	20% After 1yr	ATG, Rap/ Tac
	14	100%	71% at 1 yr 57% at ~3yr	Dac, Rap, Tac
Froud et al., 2008	3	66%	66% After 2 yr	Alemtuzumab, Rap/Tac (3 mo.), then Rap/MMF
Matsumoto et al., 2010	3	100%	53±7 days	ATG, Tac, MMF, Entercept, Anakinra
Posselt et al., 2010	3	100%	40±4 days	Dac, Rap, Tac, Entercept
	8	100%	75% at 1 yr* 38% at 2yrs*	ATG, Efalizumab, Rap, MMF
Shapiro et al., 2010	12	100%	83% at 3yrs	Alemtuzumab, Tac, MMF
Hering et al., 2011	16	88%	38%	Dac, Rap/ Tac
	8	100%	63% at 1 yr	ATG, Dac, Entercept, MMF, Rap, Tac

*These numbers are limited, because the drug being tested, Efalizumab, was withdrawn from the market during treatment.

Table 1. Summary of Clinical Islet Transplantation Outcome.

Rapamycin (sirolimus) has a different mechanism of action and side effects compared to the calcineurin inhibitors (Pritchard, 2005). Although rapamycin binds to FKBP12 in a similar mechanism like tacrolimus, it does not inhibit calcineurin, rather the complex inhibits mTOR (mammalian Target Of Rapamycin). This inhibits the second phase of T-cell activation, whereas tacrolimus and cyclosporine inhibit the first phase. So rapamycin also inhibits signal transduction leading to IL-2 production and clonal proliferation, and since it affects a different part of T-cell activation it can be used synergistically with calcineurin inhibitors. One of the side effects associated with this drug is oral ulcers that make ingestion of food difficult. Rapamycin is thus a potential alternative or adjunct to calcineurin inhibitor mediated T-cell inhibition.

Another widely used immunosuppressant is mycophenolate mofetil, MMF, which prevents B and T-cell proliferation (Ransom, 1995). This is the first of two enzymes responsible for the production of guanosine monophosphate from inosine monophosphate; lymphocytes are

particularly dependent on this pathway for the subsequent synthesis of GDP, GTP, and dGTP. The reduction of GTP and dGTP levels in lymphocytes results in a decreased ability to perform DNA synthesis and GTP dependent metabolism. Some of the adverse events associated with this drug include nausea, infections, leucopenia, and anemia. This alternative mechanism of specific immune system suppression has led to the widespread use of MMF in combination with tacrolimus or sirolimus. One such study was performed recently by Shapiro et al. in 2010 by combining MMF with tacrolimus for maintenance with alemtuzumab induction. This study was compared to the original Edmonton protocol which used daclizumab for induction and a combination of tacrolimus and sirolimus as seen in Table 1. It was hypothesized that MMF would be better tolerated than high dose sirolimus. Of the 12 patients with completed islet infusions, 83% (10/12) are still insulin independent at 36 months, whereas two patient's grafts failed after removing immunosuppressants due to infections. The mechanism underlying this tolerance seemed to be linked to a unique donor specific IL-10 regulated immune response, leading to improved insulin independence rates.

There have also been several antibody mediated approaches to preventing T-cell activation by blocking costimulation. This approach enabled a calcineurin inhibitor and glucocorticoid free immunosuppression regimen; the antibody used was efalizumab (anti-LFA 1 antibody) (Posselt et al., 2010). These antibodies were used as supplements to sirolimus/MMF maintenance therapy. The result was that 5/5 of patients receiving efalizumab achieved insulin independence with several maintaining graft function for over a year. Unfortunately, this therapy cannot be continued, because efalizumab was withdrawn from the market by the FDA in mid 2009 (Posselt et al., 2009). Despite the progress made, any use of immunosuppressants will slow the widespread applicability of islet transplantation, since the cost benefit analysis of immunosuppression must be compared to insulin therapy alone.

3.3 Xenogenic porcine islets for transplantation

Even if all pancreatic islet cell transplantations became successful and only required one pancreas to achieve insulin independence, there would still be an insufficient number of pancreata to treat all type 1 diabetic patients. There are approximately 7,000 pancreases available for donation in the United States per year, but diabetes mellitus affects as many as 3 million people in the United States and as many as 16 million people worldwide. This shortage in human islet cells from organ donor sources has led to a search for alternative sources (Hering & Walawalkar, 2009). One source that has been identified is porcine islets, which secrete physiologically functional insulin but can be highly immunogenic to humans and carry a risk of cross-species infection by pig endogenous retroviruses.

A particular surface epitope that has led to increased xenoreactivity is galactose- α 1,3-galactose (Rayat et al., 1998; Korbitt et al., 1997). Through genetic engineering of pig herds, a knockout animal which lacks this epitope has been successfully bred (Puga Yung et al., 2009). However kidney transplantation results using this animal have shown only modest gains.

Another fear of porcine islet transplantation is that pig endogenous retrovirus (PERV) could be transmitted from the porcine islets transplanted into the human recipient. Although pigs can be bred in pathogen-free facilities that shields them from contracting microbes that can be transmitted, PERV is integrated into the genome and the risk of infection cannot be so easily removed. When recipients are immunosuppressed, porcine cytomegalovirus or pig

lymphotrophic herpes virus are common infections that can be activated under immunosuppression. There have been conflicting reports of PERV manifesting in non-obese diabetic mice transplanted with pig islets. For humans, sensitive detection techniques such as quantitative polymerase chain reaction or immunoblotting assays for PERV proteins. With respect to clinical trials of xenotransplantation, there is little evidence of transmission of xenogenic endogenous virus transmission (Elliott et al., 2005; Garkavenko et al., 2011).

Porcine islets have even been used in two clinical trials of islet transplantation, one in Mexico (Valdes-Gonzales et al., 2005) and one in New Zealand (Garkavenko et al., 2011). The Mexico trial involved transplantation of collagen generating devices embedded with islet and sertoli cells into twelve juvenile type 1 diabetic patients. The purpose of the sertoli cells was to prevent the action of the immune system. This procedure did not produce long term insulin independence, but glycemic control was improved along with positive porcine C-peptide in patient urine during long term follow-up.

A second noteworthy clinical trial is being performed in New Zealand using alginate encapsulated neonatal porcine islets transplanted into the peritoneal cavity of type 1 diabetic patients. The pigs used were raised in pathogen-free facilities with a donor herd free from conventional pathogens and non-transmittable PERV. This unique lack of exposure to viruses is a characteristic of these pigs which were raised on the island of New Zealand with limited exposure to foreign diseases. In terms of xenogenic related infection, zoonosis has been rigorously monitored with no evidence of cross-species infection. Again, no patients became insulin independent, but there was a significant reduction in hypoglycemic unawareness episodes. This encapsulation method demonstrates the usefulness of immunoisolation in islet transplantation (Elliott RB, 2011).

Porcine islets have been used to explore alternative transplant sites, since blood in the portal vein of the liver often contains higher concentrations of immunosuppressants and other toxins, while containing low concentrations of oxygen. Areas of porcine islet transplantation into non-human primates include intraperitoneum, renal subcapsule, subcutaneous, the omentum pouch, and the mesentery (Hering & Walawalkar, 2009). The efficacy of each transplant site is difficult to determine as various immunosuppressive therapies were used in addition to encapsulation and the use of a subcutaneous mono-layer device. These large animal studies have shown some success demonstrating the ability in some cases to create insulin independence with immunosuppressants. Finally, genetic modification of donor pigs permits the upregulation of certain genes that would have cytoprotective or immunomodulatory effects to improve engraftment and reduce the effect of the immune response.

3.4 Islet Immunoisolation

The leading strategy to reduce or eliminate the most risky aspect of islet transplantation, namely immunosuppression drugs, is immunoisolation. To protect islets from the recognition of the immune system, two strategies have been used, specifically encapsulation and surface modification (Wilson & Chaikof, 2007) as shown in Figure 1. Encapsulation involves, as its name suggests, enclosing an islet in a capsule that is impermeable to the immune system. These capsules are typically composed of agarose or poly(vinyl alcohol) alginate. The capsules must have defined pore sizes that allow the influx of glucose as well as the secretion of insulin, while simultaneously preventing intrusion by complement or antibodies. These modifications also allow drug delivery by covalent attachment of anti-

thrombogenic or immunosuppressive agents, which would be applicable to both human allotransplantation or xenotransplantation.

3.4.1 Encapsulation

The encapsulation method is limited by bioincompatibility and biodegradation of the materials in addition to hypoxic damage to the islets (Lee & Byun, 2010). Further there is the practical limitation of size; the typical islet diameter of 150 μm is often increased by as much as 5 fold, leading to a volume increase greater than 100 fold. Since more than 500,000 islet equivalents are now required to cure diabetes (~10mL islet pellet volume), that volume would be increased to over 1 liter with encapsulated islets. This would exclude transplantation into the portal vein of the liver, because this volume of islets would significantly increase the risk of portal vein thrombosis and other bleeding complications. Thus, alternative sites of injection such as subcutaneous, intraperitoneal, or intramuscular have to be validated. However, this technique still would have the issue of incomplete encapsulation or variable number of islets entrapped.

3.4.2 Surface modification

An alternative to macroencapsulation is surface modification, which uses the islet surface as the scaffolding upon which to build protective barriers. One such surface modification method is conformal coating, which uses derivatives of the molecule diacrylate (Cruise et al., 1999) Conformal coating uses the islet surface to deposit or chemically grow a protective barrier without as large of a size increase; the final diameter is only increased by 30 to 50 μm in human and porcine islets.

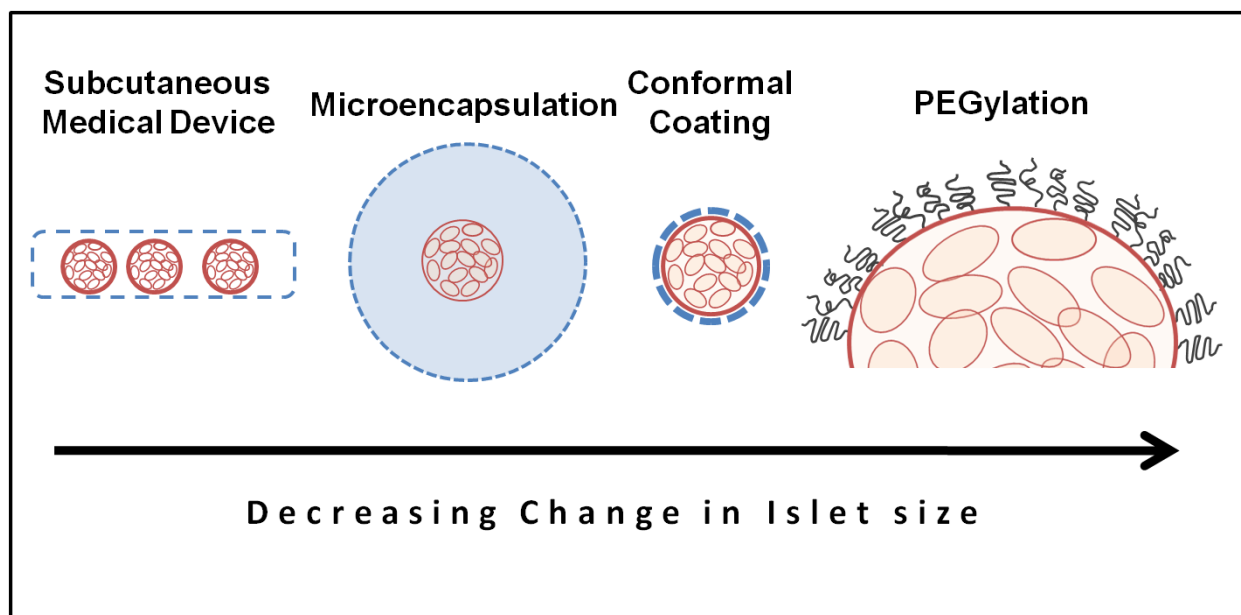


Fig. 1. Immunoisolation methods for islets from largest size to smallest increase in size.

Another study demonstrated the use of proteins as a protective barrier by reacting a disuccinimidyl bifunctional poly-ethylene glycol (PEG) molecule on one side with the islet surface and the other side to albumin (Xie et al., 2005). Another method of surface modification is PEGylation, which attaches long chains of PEG, a hydrophilic, biologically

inert, flexible polymer chain. This method of surface modification has been applied previously to the immunoisolation of red blood cells to mask the ABO surface antigens from host antibodies (Scott et al., 1997; Murad et al., 1999). These experiments show that surface PEGylation of islets can be accomplished successfully without loss in viability or functionality. One of the main advantages of PEGylation to islets is that there is no significant size increase like that associated with macroencapsulation. There is only a microscopic increase in size, because the PEG conjugated islets (PEGylated islets) are modified at the molecular level. The traditional and most widely used PEGylation technique applied uses a succinimidyl functionalized PEG (N-hydroxysuccinimide, NHS), which reacts to surface amine groups on the islet surface (Lee et al., 2007). However, there are several other surface moieties that can be targeted by chemical modification. There have been studies that investigate PEGylation by targeting the lipid membrane (Teramura et al., 2007), and surface sugars (Wilson et al., 2010). This same study also analyzed optimization of surface modification by varying reaction time and concentration of the functionalized PEG; the new oxidative method was even combined with the traditional NHS method. However there remain more surface modification targets available such as disulfide bridges; even photochemical techniques that have been applied to protein linkage of tissue (Zhang et al., 2004). Another poorly studied aspect of islet surface modification is in relation to the longevity of the modification methods. The islet surface is a dynamic environment, with constant turnover and regeneration of the cell membrane. Almost all of the PEGylation techniques mentioned only look at the uniformity and quantity of the PEG immediately after modification. If PEG is supposed to protect islets from the immune system, it should be robust and remain for a long time in order to protect islets from T-cell mediated rejection. Biologically active agents that have been attached to islet surfaces include heparin, activated protein C, urokinase, or thrombomodulin (Cabric et al., 2007; Contreras et al., 2004; Teramura & Iwata, 2008; Stabler et al., 2007). Development of a standardized analytic method that determines PEG density/ islet (size adjusted) and uniformity over a long time period would provide the ability to compare surface modification chemistries and optimize PEGylation reactions.

4. Beta cell regeneration

Development of type 1 diabetes is a consequence of loss of functional beta cell mass. Hence the objective of many therapeutic approaches to treat T1D is to restore beta cell mass sufficient to maintain normoglycemia. Results from the clinical islet transplantation trials have reinforced this concept. In a healthy individual, the beta cells lost are constantly replenished by beta cell neogenesis through mechanisms which are not clearly understood. Beta cell mass is maintained and regulated in response to pharmacological and nutritional stimuli which include glucose, insulin, epidermal growth factor (EGF), gastrin and glucagon like peptide (GLP-1). Physiological stimuli such as pregnancy, obesity and pancreatic tissue damage also significantly regulate beta cell mass. Beta cell development in pancreas is regulated by the hierarchical expression of a specific complement of transcription factor proteins (Edlund H, 1998). Originating from cells expressing homeodomain transcription factor PDX1 (pancreatic/duodenal homeobox 1) and repressing sonic hedgehog, both endocrine and exocrine cells of the adult pancreas arise from endodermal cells expressing PDX1 and Ptfla. Fibroblast growth factor family of proteins stimulate the proliferation of

progenitor cells in addition to specific stimuli from mesenchymal cells. Formation of beta cells is further driven by the transient expression of Pax4 in ngn 3-expressing cells. Beta cell expansion begins when transcription factor complement being driven by PDX1, Pax6, Nkx2.2 and Nkx6.1 (Edlund H, 1998).

In the adult pancreas, beta cell replication is a key phenomenon for the emergence of neogenic beta cells, however, the rates of beta cells replication is extremely low. The origin of neogenic beta cells is unclear. Several possibilities including differentiation of pancreatic stem cells or ductal or acinar cells are proposed. The plasticity of adult pancreatic cells is thought to play an important role in the neogenesis of beta cells. Attempts to transdifferentiate liver cells or intestinal K cells in insulin producing beta cells have also been reported through coordinated alteration in gene expression and cellular phenotype (Campbell and Macfarlane, 2007). A number of hormones and growth factors have been shown to stimulate renewal of beta cells which include GLP1 analog, EGF and gastrin. GLP1 is an incretin hormone produced by L-cells of the intestine. GLP-1 augments insulin secretion (Melloul et al, 2002). GLP-1 binds to specific GLP-1 receptor and activates intracellular signaling events involving protein kinase A and changes in cyclic AMP levels. GLP-1 released from the intestinal L-cells is rapidly degraded by the dipeptidase enzyme DPPIV. Inhibitors of DPPIV are now increasingly used to control glucose levels in type 2 diabetic patients. GLP-1 has also been shown to increase PDX1 gene expression in ductal cells during the regeneration of pancreas (Sharma et al., 1999). A similar line of action has been proposed for thiazolidinediones in the enhancement of neogenic beta cell function through activation of nuclear receptor peroxisome proliferator activator gamma (PPAR) (Richardson et al., 2006)

5. Stem cells

Stem cells have the potential to undergo symmetric cell divisions as well as asymmetric cell divisions for lineage commitment such as differentiation into insulin-producing cells. Stem cells may offer an important and unlimited source for beta cell replacement. Stem cells can be broadly classified as embryonic (ESC) or adult (ASC) stem cells. ESCs are isolated from the blastocyst which is formed during embryonic development while ASCs are detected within tissues (Fuchs et al., 2004). Both cell types are capable of commitment to specific cell lineages. ESCs exhibit greater plasticity (pluripotency) in terms of differentiation into almost any cell type, however, ASCs are limited in their commitment to repertoire of cell types (multipotency).

ESCs offer a potential source for beta cell neogenesis. However, it is difficult to maintain human ESCs in an undifferentiated state under *in vitro* culture conditions. The molecular mechanisms that derive ESCs into insulin producing are not clearly defined yet; however, nutrients, oxygen and other growth factors have been shown to play a critical role (Smith, 1991). Only a small percentage (<2%) of ESCs have been shown to spontaneously differentiate into insulin producing cells, thus limiting their clinical application. Several cell culture strategies have been proposed to increase the percentage of insulin producing cells from ESCs. Unfortunately the amount insulin obtained with these strategies is extremely low when compared to insulin content of the beta cells of pancreas (Roche and Soria, 2006). Despite their limited capacity for proliferation ASCs offer the advantage of immune compatibility in the development of beta cell replacement therapies. However, ASCs may

still be targeted by the autoimmune response. ASCs isolated from pancreatic tissue have been shown to differentiate *in vitro* into islet-like structures (Bonner-Weir et al., 2000; Ramiya et al., 2000) lending support to the notion that ductal cells may serve as islet progenitors. Culture of ductal cells isolated from human pancreas under specific conditions resulted in islet-like clusters that exhibited islet-specific hormones and transcription factors. Further, these islet-like clusters showed increased insulin response to glucose challenge. In contrast, Dor et al., (2004) have proposed that beta cell neogenesis in adult pancreas is solely derived from replication of pre-existing beta cells and not from stem cell precursors. Thus identification of true islet progenitors and the mechanisms that differentiate stem cells into normal insulin producing beta cells are still under progress.

In summary, great strides have been made in development of a physiological treatment for insulin-dependent diabetes. Four broad types of approaches as shown in figure 2 are currently being pursued to find a cure for this increasing health hazard. While some of these approaches have progressed well enough to find clinical application, others are lagging behind due to technological deficiencies. Transplantation of allogenic pancreas organ or

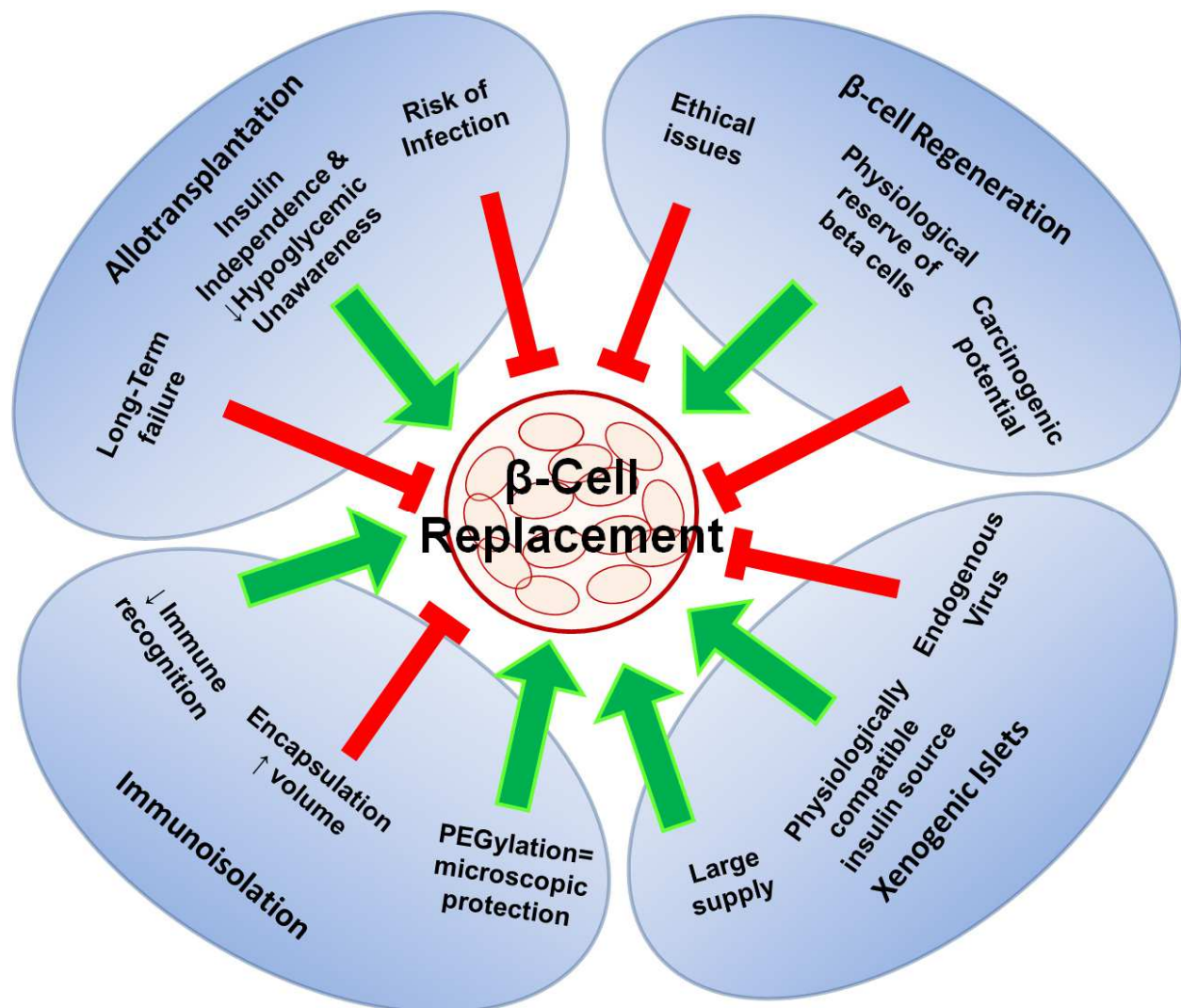


Fig. 2. Opportunities and Challenges to beta cell replacement therapies. Green colored arrows indicate opportunities and the red colored hammer indicates challenges.

isolated pancreatic islets have shown the most progress in terms of clinical application and are currently limited by the lack of suitable organ supply and islet-friendly immunosuppression. Transplantation of pig islets could overcome the hurdle of donor organ shortage, but the safety and efficacy of such xenotransplantation remains to be determined. Immune isolation of allogenic and xenogenic islets will significantly prolong the survival of transplanted islets and also prevent the harmful effects of host immune response. Optimization of encapsulation technologies to support islet cell function in the long term is not fully developed yet. Replacement of beta cells derived from alternative sources such as stem cells has the potential to offer unlimited supply of beta cells and is currently receiving greater attention from the research community.

6. Conclusion

Incidence of diabetes is increasing at an alarming rate in different populations all over the world. Diagnosis of T1D is also following this increasing trend, constituting 5-10% of the total diabetes cases. Exogenous insulin therapy is proving to be adequate for majority of T1D patients; however, for “brittle” T1D patients control of blood glucose levels is very difficult using this treatment. Transplantation of whole organ pancreas is an established procedure to restore beta cell mass to attain normoglycemia. Transplantation of isolated islets has seen a tremendous growth in the past decade in terms of the number of transplant recipients, improvement in the islet isolation methodologies and post-transplant graft survival and function. Due to limited supply of qualified pancreata for transplantation, other approaches to replace beta cells are gaining attention. Pig islets are an attractive option in terms of abundant supply and physiological similarity of insulin despite the possible risk of infection. Beta cell replacement using regeneration of stem cells or other cell types have shown promising results, however, are too far away from clinical application. Importantly, immune isolation of transplanted islets or beta cells using macro- or micro-encapsulation technology may significantly improve the current outcomes of cell-based therapy.

7. Acknowledgement

This work was supported by a grant (#5-2010-668) from the Juvenile Diabetes Research Foundation (to B.N.) and by the Baylor Health Care System. JAS is supported by a fellowship from Baylor University Institute of Biomedical Studies, Waco, Texas. The authors thank Ana Rahman and Yoshiko Tamura for their help in preparation of the manuscript.

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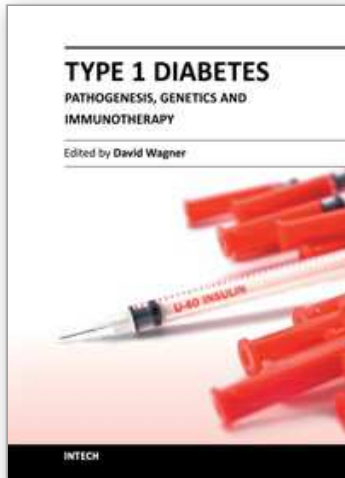
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Type 1 Diabetes - Pathogenesis, Genetics and Immunotherapy

Edited by Prof. David Wagner

ISBN 978-953-307-362-0

Hard cover, 660 pages

Publisher InTech

Published online 25, November, 2011

Published in print edition November, 2011

This book is a compilation of reviews about the pathogenesis of Type 1 Diabetes. T1D is a classic autoimmune disease. Genetic factors are clearly determinant but cannot explain the rapid, even overwhelming expanse of this disease. Understanding etiology and pathogenesis of this disease is essential. A number of experts in the field have covered a range of topics for consideration that are applicable to researcher and clinician alike. This book provides apt descriptions of cutting edge technologies and applications in the ever going search for treatments and cure for diabetes. Areas including T cell development, innate immune responses, imaging of pancreata, potential viral initiators, etc. are considered.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Jeffrey A. SoRelle and Bashoo Naziruddin (2011). Beta Cell Replacement Therapy, Type 1 Diabetes - Pathogenesis, Genetics and Immunotherapy, Prof. David Wagner (Ed.), ISBN: 978-953-307-362-0, InTech, Available from: <http://www.intechopen.com/books/type-1-diabetes-pathogenesis-genetics-and-immunotherapy/beta-cell-replacement-therapy>

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