

Angiogenic gene therapy for improving islet graft vascularization

Review Article

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Key words: Type 1 diabetes, islet transplantation, islet revascularization, VEGF, gene transfer.

Received: 3 July 2003; Accepted: 19 August, 2003; electronically published: August 2003

Summary

Clinical islet transplantation is considered a curative treatment for type 1 diabetes, but long-term survival and function of implanted islets is greatly compromised by a number of adverse events. In addition to immune rejection and recurrent autoimmunity, the survival and function of islets is determined by the rate and degree of islet revascularization, an essential process termed angiogenesis that is required for the development of new vessels within islet grafts to derive blood from the host vasculature. Rapid and adequate revascularization is crucial for islet survival and function. Delay in islet revascularization can deprive islets of oxygen and nutrients, resulting in islet cell death and early graft failure. There is evidence that despite the infusion of sufficiently large amounts of islets (~11,000 islets/kg body weight) per diabetic recipient, less than 30% of islet mass becomes stably engrafted post transplantation. In this article, we will review the molecular basis of islet revascularization and highlight the importance of developing novel therapeutic strategies to stimulate angiogenesis within islet grafts and enhance islet graft vascularization post transplantation. Such strategies, when applied in conjunction with islet transplantation, are expected to improve the viability of transplanted islets and provide long-term survival of functional islet mass post transplantation, thereby increasing the overall success rate of islet transplantation.

I. Introduction

A. Type 1 diabetes

Type 1 diabetes is a metabolic disorder that is caused by insulin deficiency due to autoimmune destruction of cells, leading to chronic elevation of blood sugar levels. Because of its onset in children and young adolescents, type 1 diabetes was previously referred to as juvenile diabetes or insulin-dependent diabetes. Prior to the discovery and isolation of insulin for therapeutic use, patients with type 1 diabetes survived only for a period of months, with death caused primarily by the accumulation of ketones in the body, leading to diabetic ketoacidosis. Over the past century, the prevalence of type 1 diabetes has increased in a variety of populations with an incidence rate ranging from 1-3 per 100,000 children per year in the US at the beginning of the 20th century to 4-7 per 100,000 in Scandinavian countries between 1930-1950, and to approximately 20 per 100,000 in Scandinavia over the past two decades (Bloomgarden, 1998; Gale, 2002). Currently, there are about 1.7 million patients with an overall annual

incidence of about 15 per 100,000 children in the US alone (Karvonen et al, 2000). This poses a tremendous burden on patients and healthcare economies.

B. Insulin therapy and limitations

Type 1 diabetes is commonly treated with twice-daily injection of a mixture of delayed and short-acting insulin. Delayed-acting insulin is provided to maintain a relatively constant background level of plasma insulin for the basal requirement, on which short-acting insulin is imposed to meet the postprandial demand of insulin after meals. Nevertheless, such conventional insulin therapy typically leads to inadequate blood sugar control as most treated patients experience to a lesser or greater extent elevated blood sugar levels between meals and during the night, the cumulative effect of which can result in the development of diabetic complications at a late stage. There is clinical evidence that more than half of diabetic patients have eyes affected by diabetic retinopathy (Bloomgarden, 1998), with additional effects on the

kidneys by diabetic nephropathy (Chaturvedi et al, 2000) and on nerves by diabetic neuropathy, together with about 4- and 10-fold lifetime increase in rates of cardiovascular mortality among men and women, respectively (Laing et al, 2003). To improve glycemic control, a number of insulin analogs, such as short-acting insulin lispro and aspart (Plank et al, 2002), as well as delayed-acting insulin glargine (Murphy et al, 2003) and detimir (Vague et al, 2003) have been developed. Nevertheless, implementation of treatment regimens with insulin analogs in different formulations to strive for normoglycemic control without risk of hypoglycemia can be very challenging and requires extraordinary efforts from both health care providers and diabetic patients (Bloomgarden et al, 2002).

C. Islet transplantation

Of alternative insulin replacement therapies developed, islet transplantation offers the prospect of providing a curative treatment for type 1 diabetes without the need for exogenous insulin. The protocol of islet transplantation developed by Shapiro and colleagues at the University of Alberta at Edmonton, Canada, known as the Edmonton protocol, is relatively simple and minimally invasive, which is carried out under local anesthetics without surgery. Using fluoroscopic guidance, isolated human islets are implanted intraportally to a diabetic recipient, such that islets are engrafted in the liver and function to provide near physiological insulin release from an ectopic site. The success of this protocol has largely been attributed to technical advances in isolating high-quality human islets in relatively large quantities and the application of more potent and less toxic non-steroidal immunosuppressants (Shapiro et al, 2000). Using the Edmonton protocol, long-term excellent glycemic control has been achieved with sustained freedom from insulin injection in type 1 diabetic patients (Shapiro et al, 2000). Currently, this protocol is being rigorously tested in clinical trials at multiple clinical centers to evaluate the safety and efficacy of islet transplantation and assess the benefit and risk ratio associated with long-term use of immunosuppressive drugs (Boker et al, 2001).

Although promising for providing a curative option for type 1 diabetes, the Edmonton protocol is limited by two major factors: the lack of a sufficiently large source of islets due to the scarcity of cadaveric pancreas donors, and the presence of persistent immune rejection as well as the potential for recurrence of autoimmunity. Recent follow-up studies indicate that even with the rigorous application of steroid-free immunosuppressive regimens, there is still a slow and progressive loss of insulin production from transplanted islets in diabetic recipients over time, as evidenced by reports that 30-40% of islet recipients may experience recurrence of autoimmune diabetes with re-acquisition of insulin dependence one to two years post transplantation (Shapiro et al, 2000; Boker et al, 2001; Ryan et al, 2001, 2002). To overcome these limitations, attempts have been made to develop alternative islet

sources by generating insulin-producing cells through genetic engineering of embryonic stem cells (Lumelsky et al, 2001; Soria et al, 2001). In addition, limited progress has been made to induce graft tolerance using immune modulation or allorecognition (Cote et al, 2001). An in-depth discussion of these two outstanding issues in relation to the optimal clinical outcome of islet transplantation, which is beyond the scope of this article, has been reviewed elsewhere (Waldmann, 2002; Lechler et al, 2003; Lechner and Habener, 2003). Here we would like to highlight a third limiting factor, namely islet revascularization, which appears to play an important role in determining the long-term survival and optimal performance of functional islet mass post transplantation.

1. Islet revascularization post transplantation

a. Re-establishment of islet microvasculature.

Native islets in the pancreas have a rich glomerular-like vascular system that consists of fine capillaries supplied by one to five feeding arterioles and drained by coalescing into an efferent plexus exiting the islet via one to five venules (Menger et al, 2001; Mattson et al, 2002). Such a rich microvasculature in pancreatic islets serves to provide efficient delivery of oxygen and nutrients to islet cells, and at the same time ensure rapid dispersal of pancreatic hormones to the circulation. However, isolated islets are avascular in both structural and functional entities, such that after transplantation, the survival and function of islets must rely on the re-establishment of new vessels in the grafts to derive blood flow from the host vessel system (Boker et al, 2001; Vasir et al, 2001). There is evidence that freely transplanted islets are associated with significantly reduced islet revascularization in comparison to native islets in the pancreas and this problem occurs irrespective of whether islets are transplanted intraportally in the liver, retrogradely into the spleen, or under the kidney capsule (**Figure 1**) (Mattson et al, 2002).

What are the likely consequences of delayed or insufficient islet revascularization post islet transplantation? To answer this question, let us take a quantitative view of the relative partitioning of blood flow to islets vs. exocrine tissue in the pancreas. Using a modified microspheres technique, it has been shown that islets take up more than 10% of the total pancreatic blood flow despite their collectively comprising only about 1% of the tissue mass of the pancreas (Jansson and Carlsson, 2002). Thus, it is critically important to maintain adequate microvascular perfusion to islet cells for oxygen and nutrient supplies. While islets are transplanted either as single entities or as aggregated islet clusters under the kidney capsule or intraportally in the liver, adequate microvascular perfusion to islet cells does not resume immediately after islet transplantation.

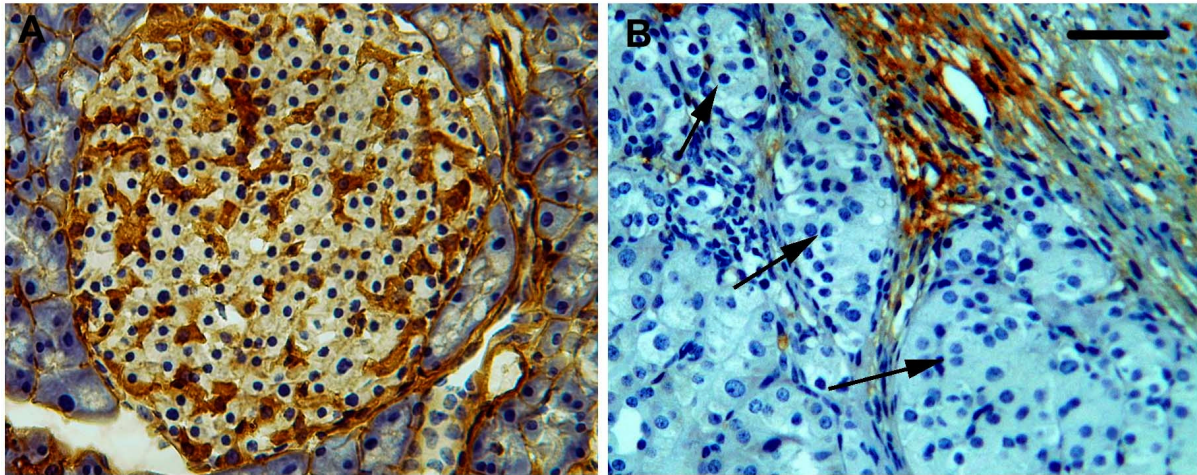


Figure 1. Intra-islet microvasculature. **A.** Microvasculature in the mouse pancreas, as visualized by immunostaining for the endothelium marker CD-31, also known as the platelet endothelial cell adhesion molecule-1 (PECAM-1). **B.** Microvasculature in engrafted islets under the renal capsule of a diabetic mouse following 16 days of islet transplantation. Islet grafts are indicated by arrows. Bar, 50 μ m.

Instead, it can take up to three to five days for the formation of intra-graft microvessels to occur post islet transplantation and the re-establishment of intra-graft blood perfusion can take even longer time (>14 days) (Vasir et al, 2001, Jansson and Carlsson, 2002). This delay in the re-establishment of a functional microvasculature in newly grafted islets can starve islet cells of oxygen and nutrients. Indeed, several studies have shown that newly transplanted islets are hypoxic, causing islet cells to undergo apoptosis and/or necrosis, which attributes to the loss of functional β -cell mass post transplantation (Vasir et al, 2001; Jansson and Carlsson, 2002).

Consistent with this interpretation, it has been shown that despite the administration of a large number of islets (11,000 islets/kg body weight) per diabetic recipient, only about 30% of transplanted islets become stably engrafted, corresponding to a total loss of about 70% of the functional islet mass in the early post transplantation phase (Boker et al, 2001). In addition, recent clinical data indicate that even when fasting blood glucose levels are restored to the physiological range post islet transplantation, the optimal performance of engrafted islets in terms of glucose-inducible insulin secretion is abnormal. In response to intravenous glucose infusion, the amplitude of the first phase insulin secretion is only about 20% of normal, which coincides with relatively slow glucose disposal rates following an oral glucose load in post-transplant subjects (Ryan et al, 2002). Although there is no direct proof suggesting that this observed suboptimal performance of transplanted islets in glycemic control is associated with insufficient vascularization, there is general agreement that impaired islet revascularization does adversely affect the optimal function of islets post transplantation. Recent preclinical studies have shown that even after transplanted islets are stably engrafted, the extent of vascularization, defined as microvascular density in transplanted islets is significantly lower than that in native islets in the pancreas (Jansson and Carlsson, 2002). In addition, engrafted islets in all three of the different transplantation organs (kidney cortex, liver and spleen) also exhibit markedly low oxygen tension, in comparison

to native islets in the pancreas, which is associated with a concomitant reduction in intra-graft blood perfusion (Carlsson et al, 2000, 2001). Currently, the extent to which this observed low oxygen tension and reduced blood perfusion in islet grafts, as a result of insufficient islet revascularization, adversely affect the long-term survival and optimal performance of functional islet mass and contribute to early graft failure is not known. An additional factor that might contribute to the metabolic abnormality in glucose tolerance in diabetic recipients is islet graft reinnervation post transplantation. However, little is currently known about its molecular basis in relation to islet revascularization and the optimal performance of islet function in glycemic control post transplantation.

b. Mechanism of islet graft vascularization

To date, the molecular mechanism of islet revascularization post islet transplantation remains poorly understood. In general, tissue graft vascularization depends on a coordinated process of angiogenesis and vasculogenesis, which are functionally governed by two key protein factors, vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang-1). These two angiogenic/vasculogenic factors play separate but complementary roles in the de novo formation of blood vessels during embryonic development (vasculogenesis) as well as in the formation of new blood vessels from pre-existing ones (angiogenesis) (Yancopoulos et al, 2000). VEGF acts in the early phase to stimulate the formation of primitive vascular networks by vasculogenesis and angiogenic sprouting, whereas Ang-1 functions subsequently for remodeling and maturation of the primary vascular system by integrating the endothelial cells of vessels with surrounding matrix and supporting cells (smooth muscle cells and pericytes) (Thurston et al, 1999). Thus, in terms of their specific roles in angiogenesis/vasculogenesis, VEGF seems to be a critical "driver" for initiating vascular formation, whereas Ang-1 works as a "stabilizer" to ensure subsequent maturation

and stability of the newly formed blood vessels. These two factors act synergistically to ensure new blood vessel formation, growth and maturation.

VEGF has four different isoforms in humans, consisting of 121, 165, 189 and 206 amino acid residues, all of which are generated by alternative splicing of a single gene. Rodents have only three isoforms, namely VEGF120, VEGF164 and VEGF188, each polypeptide one amino acid shorter than their corresponding human homologues (Kim et al, 2000; Vasir et al, 2000, 2001). The most abundant and widely distributed form is VEGF165 in humans (or VEGF164 in rodents). In concert with their respective functions in angiogenesis / vasculogenesis, the receptors for both VEGF (VEGFR-1/Flt1 and VEGFR-2/Flk-1/KDR) and Ang-1 (Tie2) are selectively expressed in the vascular endothelium (Ferrara and Davis-Smyth, 1997; Otani et al, 1999; Kim et al, 2000). In addition, both VEGF and Ang-1 are expressed in the pancreas, suggesting their functional importance in pancreatic tissue angiogenesis / vasculogenesis (Vasir et al, 2001). However, due to limited data in the literature, little is known about the functional interplay between VEGF and Ang-1 in islet revascularization post transplantation.

c. Genes involved in islet revascularization

Of the genes whose functions are involved in angiogenesis, VEGF seems to play a crucial role in islet revascularization. Recent studies by Vasir and colleagues (2000, 2001) indicate that VEGF expression in islet cells is transiently induced, followed by significant decline two-three days post transplantation. This impaired expression of VEGF is further pronounced in the presence of prevailing hyperglycemia, which coincides with delayed expression profiles of VEGF receptor molecules, Flk-1/KDR and Flt-1, in islet grafts post transplantation in diabetic animals (Hellerstrom et al, 1998; Korsgren and Jansson, 1989; Mattson et al, 2002). These results reflect to some extent an impaired angiogenesis of islet grafts in the diabetic milieu, which is contributable to the lack of adequate islet revascularization under hyperglycemic conditions.

In addition to VEGF, there are a number of other angiogenic molecules whose expression in islet cells also seems to affect islet revascularization, including fibroblast growth factor (FGF), hepatic growth factor (or scatter factor) (HGF/SF) and its receptor c-Met, transforming growth factor- β (TGF- β) and - α (TGF- α), and urokinase plasminogen activator (uPA) and its receptor uPAR. Like VEGF, FGF appears to be a positive regulator of angiogenesis, as it has been shown to induce endothelial cell proliferation, migration and angiogenesis (Bikfalvi et al, 1997; Vasir et al, 2000, 2001, Kawakami et al, 2001). Regarding the function of TGF in angiogenesis, TGF- β has been shown to stimulate the growth of microvascular endothelial cells (Tokuda et al, 2003). In addition, TGF- β is also a potent inducer of VEGF (Gille et al, 1997; Li et al, 2003). On the other hand, TGF- α is found to stimulate wound healing and regulate differentiation of certain cell

types (Chegini, 1997; Asplin et al, 2001; Li et al, 2003). Although FGF and TGF have been implicated to play important roles in angiogenesis (Vasir et al, 2000; Kawakami et al, 2001), their functional contributions to islet revascularization remain unknown.

HGF/SF is a mitogen that acts to stimulate cell division and proliferation of a variety of cell types, including smooth muscle cells and pericytes that are functionally involved in blood vessel formation (Bussolino et al, 1992; Ahmet et al, 2003; Ding et al, 2003; Sengupta et al, 2003). In addition, it has recently been shown that elevated HGF production in islet grafts significantly improves the outcome of marginal islet transplantation due to its proliferative effect on islet cells (Garcia-Ocana et al, 2003). c-Met is a tyrosine kinase receptor of HGF/SF, which is expressed in endothelial cells. In concert with the action of HGF/SF, the islet-specific expression of c-Met functions to mediate the mitogenic effect of HGF/SF on islet cell growth and proliferation (Weidner et al, 1993; Rosen et al, 1997). Vasir and colleagues (2000) showed that the expression of HGF/SF together with its receptor in newly transplanted islets is profoundly delayed in diabetic animals (Laing et al, 2003), which correlates with reduced islet graft vascularization. Nevertheless, its specific role in islet revascularization has not been defined.

The urokinase plasminogen activator system, consisting of uPA and uPAR, plays a pivotal role in angiogenic sprouting. uPA binds to its cell surface receptor uPAR and converts plasminogen to plasmin, a serine protease with a broad specificity that functions to catalyze the degradation of extracellular matrix/basement membrane, an essential process that is required for clearing a path to facilitate endothelial cell migration and tissue remodeling in an angiogenic cascade (Saksela and Rifkin, 1988; Bacharach et al, 1992; Pepper et al, 1993). Consistent with their roles in angiogenesis, both uPA and uPAR expression are stimulated by VEGF and HGF/SF (Pepper et al, 1992; Mandriota et al, 1995). Like other angiogenic molecules, the expression of uPA and uPAR in newly engrafted islets is significantly delayed (Vasir et al, 2000). It has been suggested that impaired uPA and uPAR expression in newly transplanted islets also contributes to insufficient islet revascularization under diabetic conditions.

4. Factors affecting islet revascularization

As discussed above, islet revascularization is an important determinant for the clinical outcome of islet transplantation. Unfortunately, transplanted islets are invariably associated with markedly reduced revascularization no matter whether islets are transplanted in the renal, splenic or hepatic subcapsular space (Jansson and Carlsson, 2002). What are the factors that adversely affect islet revascularization?

One potential factor that affects islet revascularization is the presence of prevailing hyperglycemia in diabetic recipients. Data in support of this view have been obtained by Vasir et al. (2000, 2001), who showed that the expression of several key angiogenic

proteins and their respective receptor molecules in newly engrafted islets is significantly delayed in diabetic recipient mice, compared to that in nondiabetic recipient mice. These results suggest that islets transplanted under the renal capsule in a diabetic environment fare less well in terms of graft vascularization than those transplanted in a normoglycemic subject. In contrast, a different view of the possible impact of prevailing hyperglycemia on islet revascularization is provided by Menger et al, (1992), who showed that the relative microvascular blood perfusion is equivalent in islets engrafted in the striated skin muscle in hyperglycemic and normoglycemic Syrian golden hamsters. Unfortunately, there is no quantitative data regarding the functional vascular density in islet grafts in relation to the presence or absence of persistent hyperglycemia provided in these studies. Thus, whether and to what extent prevailing hyperglycemia affects islet revascularization still remain an issue of debate.

A second factor that may potentially influence islet revascularization is the use of immunosuppressive agents associated with islet transplantation. One outstanding concern is that immunosuppressive agents are commonly associated with anti-proliferative activity and their clinical application in conjunction with islet transplantation may adversely affect islet revascularization. The immunosuppressants, sirolimus and tacrolimus, are shown to inhibit angiogenesis in a dose-dependent manner in both *in vitro* and *in vivo* angiogenesis assays (Eckhard et al, 2003). In the same sensitive assays, cyclosporine and prednisolone are also found to retain anti-angiogenic activities in counteracting the proliferative effect of FGF in angiogenesis (Eckhard et al, 2003), although it has been previously reported that the application of cyclosporin-A does not seem to alter microvascular perfusion to islet grafts (Mendola et al, 1997; Vajkoczy et al, 1999). These results raise a great deal of concern that clinical application of immunosuppressive drugs, which is intended to prevent islet graft loss, may actually compromise the viability of newly transplanted islets by hampering the process of islet revascularization.

A third limiting factor for islet revascularization is the presence of contaminating exocrine cells in isolated islets, including macrophage, dendritic cells (DC) and endothelial cells. It has been suggested that exocrine cells perturb angiogenesis and islet revascularization (Heuser et al, 2000; Jansson and Carlsson, 2002). Consistent with this idea is the observation that culturing of islets prior to transplantation tends to improve the outcome of islet transplantation, as culturing helps eliminate contaminating cells, in particular, the antigen presenting cells (APC) in islet preparation (Gaber et al, 2001; Kuttler et al, 2002). However, culturing of freshly isolated islets also results in the loss of endothelium in islets. Interestingly, recent studies show that intra-islet endothelial cells serve as integrated components in angiogenesis and function together with recipient endothelium to facilitate the overall islet graft vascularization (Brissova et al, 2003; Linn et al, 2003). These results suggest that transplantation of freshly isolated islets may be favorable for islet viability because of the functional contribution of intra-islet endothelial

cells to islet revascularization post transplantation (Jansson and Carlsson, 2002).

Finally, a less well-characterized factor that might affect islet revascularization is islet cryopreservation. This process is necessary as it can afford a great deal of flexibility and additional advantages to clinical islet transplantation. Cryopreservation allows pooling of marginal islets and subsequent distribution of islets to different islet transplantation centers/hospitals. It also allows sufficient time for pre-transplantation quality control testing of isolated islets to ensure islet cell viability and microbiological sterility prior to transplantation. In addition, cryopreservation also allows for genetic modification of islets by introducing angiogenic, cytoprotective or immunomodulatory genes via gene transfer to islets prior to islet transplantation to improve the clinical outcome of islet transplantation in the future. However, recovery of functional islets after cryopreservation has been technically challenging, as freezing and thawing can significantly reduce the viability of islet cells (Kuo et al, 2002). Up to 50% of functional islet loss has been reported after cryopreservation (Lakey et al, 2001). Furthermore, the extent to which cryopreservation affects islet revascularization remains to be determined.

B. Enhancing islet revascularization

1. Angiogenic gene transfer to enhance islet revascularization

As discussed above, rapid and sufficient islet revascularization is crucial for long-term survival and function of islet grafts post transplantation. Delayed and inadequate revascularization of newly transplanted islets can deprive islet cells of oxygen and nutrients, resulting in islet cell death and premature graft failure. Given the fact that successful islet transplantation depends on the infusion of sufficiently large amounts of islets, which usually requires at least two cadaveric pancreata per recipient, increased islet revascularization is expected to reduce the number of islets and improve the pancreas donor to recipient ratio required for transplantation. In addition, rapid and adequate islet revascularization will protect islet grafts from hypoxia-induced inflammation and necrosis, thereby improving long-term graft survival and providing better preservation of functional islet mass. However, only limited efforts have been made in the past in this aspect of islet transplantation.

VEGF is known to play a pivotal role in angiogenesis / vasculogenesis. To investigate its angiogenic effect on islet revascularization, Sigrist and colleagues (2002) have applied collagen-immobilized VEGF protein in encapsulated islets, followed by transplantation into the peritoneal cavity of streptozotocin-induced diabetic mice. Blood glucose and plasma insulin levels were determined and animals were sacrificed two weeks post transplantation. It was found that islets transplanted in the presence of collagen-immobilized VEGF protein show significantly increased angiogenesis and microvasculature in islet grafts, which associated with increased insulin production and improved glycemic

control, in comparison to control islets that are transplanted in the absence of VEGF protein. These results suggest that local VEGF delivery to islet grafts improves the outcome of islet transplantation by enhancing islet revascularization (Sigrist et al, 2002).

To improve islet graft vascularization, we have delivered the human vascular endothelial growth factor (hVEGF) cDNA by adenoviral-gene transfer to mouse islets, followed by transplantation under the renal capsule in streptozotocin-induced diabetic mice (Zhang et al, 2003). We showed that all the renal capsules containing the hVEGF vector-transduced islets (250 islets) displayed significantly higher functional islet mass, as measured by insulin immunostaining, and greater vascular density, as determined by immunostaining of CD31, the platelet endothelial cell adhesion molecule-1 (PECAM-1) (Watanabe et al, 2000). As a result, diabetic mice receiving the hVEGF vector-treated islets exhibited normoglycemia with improved glucose tolerance. In contrast, diabetic mice receiving an equivalent islet mass that were pre-transduced with a control vector maintained moderate hyperglycemia with impaired glucose tolerance. These results provide the proof-of-principle that angiogenic gene transfer to islets prior to islet transplantation allows local production of VEGF in islet grafts, which in turn stimulates graft angiogenesis and augments islet revascularization (Zhang et al, 2003).

While therapeutic angiogenesis, so called biobypass, has been considered an alternative modality for treating coronary and peripheral artery diseases, based on the efficacy and safety of plasmid- or adenoviral vector-mediated VEGF delivery in angiogenesis in a number of preclinical studies and clinical trials (Isner, 2002; Koransky et al, 2002; Mercadier and Logeart, 2002; Rasmussen et al, 2002; Sylven, 2002, Khan et al, 2003; Kusumanto et al, 2003), our view is that a similar

angiogenic strategy should be explored to accelerate islet graft angiogenesis, allowing rapid and adequate islet revascularization post transplantation. Such an approach, when used in conjunction with islet transplantation, has the potential for improving the success rate and clinical outcome of islet transplantation with long-term glycemic control at a reduced cost of islets.

2. Ex vivo gene delivery to islets

The rationale for enhancing islet graft vascularization by angiogenic gene transfer is as follows: islets are transduced in culture with a vector expressing angiogenic molecules, such as VEGF, followed by transplantation into a diabetic subject, as illustrated schematically in **Figure 2**. Using an adenoviral-mediated gene delivery system, we have validated this concept by showing that VEGF production in newly transplanted islets significantly improves islet revascularization and functional islet mass (Zhang et al, 2003). It is noteworthy that adenoviral vectors are associated with immunogenicity. In addition, islets are terminally differentiated post-mitotic cells, which poses a great challenge for ex vivo gene delivery to islets by vectors whose transduction depends on cell division (Ito and Kedes, 1997; Robbins and Ghivizzani, 1998). However, recent advances in both viral and nonviral vector development have made it feasible to transfer genes to intact islets ex vivo at reasonable efficiencies without adversely affecting the architecture and function of islets. Below is a focused review of a number of vector systems that are currently in use for ex vivo gene transfer to isolated islets.

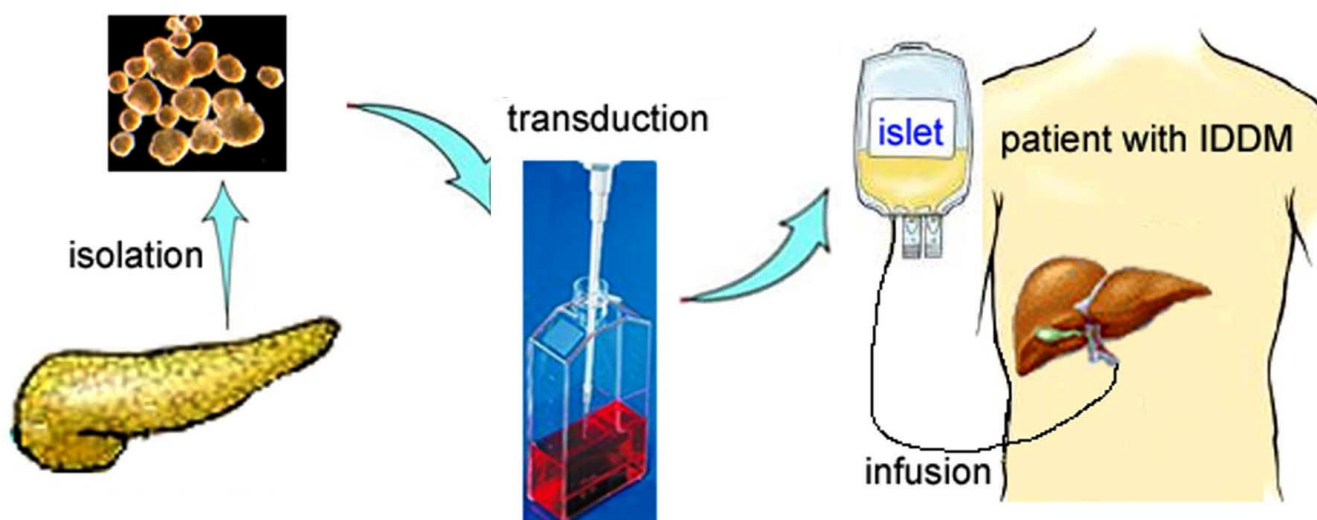


Figure 2. Schematic representation of angiogenic gene transfer in conjunction with islet transplantation. Islets are isolated and incubated in culture media in the presence of a gene vector that expresses angiogenic molecules. After transduction, islets are transplanted intraportally into the liver of a diabetic subject.

a. Adenovirus-mediated gene transfer to islets

Adenovirus is the most commonly used vector system in preclinical studies due to its relatively high transduction efficiency for both dividing and nondividing cell types. Adenovirus is capable of accommodating large DNA inserts and can be produced in a large quantity and at a relatively high titer. Although adenoviral vectors have been shown to efficiently transduce islets without altering glucose-inducible insulin secretion from β cells (Newgard, 1994; Csete et al, 1995; O'Brien et al, 1999), recent studies indicate that adenoviral-mediated transduction of islets induces the production of a number of chemokines and their respective receptors, resulting in subsequent recruitment of inflammatory cells to islet grafts. This may potentially impair islet engraftment (Zhang et al, 2003).

b. rAAV-mediated gene delivery to islets

Recombinant adeno-associated virus (rAAV) has become the vector of choice for gene transfer to a variety of cell types because of its ability to mediate long-term transgene expression in the absence of cytotoxicity (Flotte et al, 2001; Kapturczak et al, 2002; Mah et al, 2002; Vizzardelli et al, 2002). The most commonly used rAAV is derived from AAV-2, an AAV serotype that belongs to a group of non-pathogenic human parvoviruses. AAV-2 contains a 4.7-kb single-stranded genome encoding viral replication (rep) and capsid (cap) genes flanked by inverted terminal repeat sequences (ITRs) (Srivastava et al, 1994). Productive replication of AAV-2 depends on adenoviral or herpes viral helper functions, in the absence of which, AAV2 establishes a "rep-dependent" latent infection by integrating its genome site-specifically into the AAVS1 site in human chromosome 19 (Kotin et al, 1992; Rabinowitz and Samulski, 1998). In rAAV-2 vectors, the entire viral coding sequences are replaced with the therapeutic gene of interest (insertion size <4.7 kb) between the two ITRs. High titer infectious viral particles are produced using an "adenovirus helper-free" system by co-transfecting a permissive cell line with the rAAV-2 shuttle plasmid and plasmids that provide the necessary helper functions as well as the Rep and Cap proteins (Kay et al, 2001). Because of the lack of immunogenicity coupled with its non-pathogenic property, rAAV-2 has not been associated with toxicity and immune response in preclinical studies and clinical trials (Kay et al, 2001).

Although rAAV-2 is able to transduce both dividing and non-dividing cells, its transduction efficiency varies significantly among different cell types (Kay et al, 2001; Qing et al, 2003). While both muscle and brain cells are efficiently transduced, only about 5% of hepatocytes can be transduced. In addition, several cell types, including murine fibroblasts and human leukemia cells, are refractory to rAAV-2 transduction (Hansen et al, 2001). This observed variability in rAAV-2 mediated transduction of different cell types is associated with the heterogeneity of cell surface receptors that are required for viral entry (Srivastava et al, 2002). To improve viral infectivity and expand AAV tropism to non-permissive cells, chimeric AAVs carrying different cell-specific ligands in their capsid proteins have been shown to transduce cells that were previously refractory to rAAV-2

transduction (Girod et al, 1999; Wu et al, 2000). Using a rAAV-5 serotype vector, Flotte et al, (2001) showed that efficient transduction of isolated murine islets could be achieved with a 100-fold lower multiplicity of infection (MOI) than rAAV-2. More recently, rAAV-2 has been pseudotyped with capsids of any one of the eight known serotypes of AAV (Gao et al, 2002; Rabinowitz et al, 2002). In these recombinant rAAV vectors, the gene of interest is inserted between the AAV-2 ITRs and packaged into the serotype-specific capsids varying from AAV-1 to AAV-8. In this way, rAAV-2 pseudotyped with AAV-1 and AAV-5 or AAV-8 capsids is shown to transduce skeletal muscle and liver at a significantly higher efficiency than the native rAAV-2 (Gao et al, 2002; Mingozi et al, 2002; Walsh et al, 2003). Using a rAAV vector encoding the green fluorescent protein (GFP), we showed that rAAV-1 and rAAV-2 are able to effectively transduce murine and human islets in culture, respectively (**Figure 3**).

c. Lentivirus-mediated gene transfer to islets

Lentiviruses are related to retroviruses, but unlike retroviruses, lentiviral vectors retain the ability to efficiently transduce non-dividing cells, although cell cycle activation has been shown to improve significantly the efficiency of lentiviral-mediated transduction (Vigna and Naldini, 2000; Chang and Gay, 2001). Using a reporter gene expression system encoding either green fluorescent protein or β -galactosidase, lentivirus-mediated gene transfer is shown to result in sustained transgene expression in a variety of quiescent cell types including pancreatic endocrine cells (Ju et al, 1998; Giannoukakis et al, 1999; Leibowitz et al, 1999; Curran et al, 2000, 2002). Recently, a lentiviral-mediated gene transfer system, derived originally from feline immunodeficiency virus (FIV) (Wang et al, 1999), has been developed. The tropism of FIV is feline-specific with suggestive evidence of safety in humans, as veterinarians bitten and scratched by FIV-infected cats do not display signs of sero-conversion or disease (Djalilian et al, 2002).

FIV can mediate stable transgene expression because its chromosomal DNA is integrated into the host genome. In the literature, FIV-mediated transgene expression persisting for up to 6 months in vivo has been reported (Wang et al, 1999; Hughes et al, 2002). To test the ability of FIV to transduce islet cells, we have used the FIV-LacZ vector to transduce freshly isolated murine islets, demonstrating that FIV is effective in transducing islets in culture (**Figure 4**). Furthermore, FIV-mediated transduction of islets does not perturb islet function, as the characteristic feature of glucose-inducible insulin secretion from β cells remains unchanged before and after FIV transduction (Zhang et al, 2002). Our results are consistent with Curran et al. who recently showed that FIV vectors efficiently transduce human and murine islets in vitro (Curran et al, 2002).

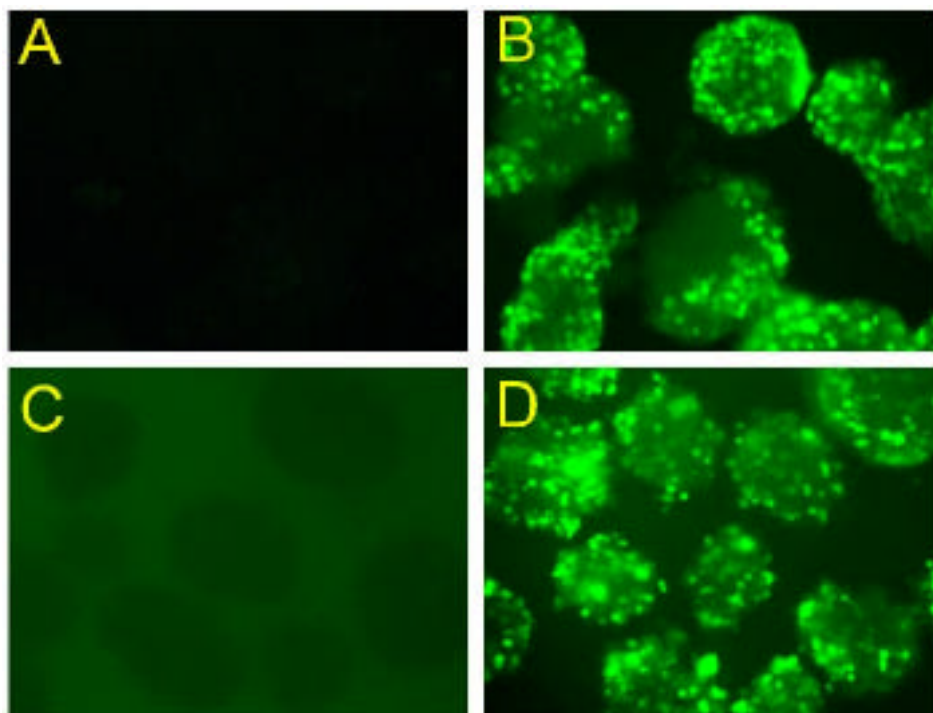


Figure 3. *Ex vivo* transduction of murine and human islets by rAAV. Prior to exposure to rAAV, islets were incubated with a helper adenovirus (Adv-5) at an MOI of 5 pfu/cell for 2 h in CMRL-1066 medium (Sigma-Aldrich, St. Louis, MO) in a 37 °C incubator with 5% CO₂. Subsequently, islets were transduced with the rAAV-GFP vector expressing the green fluorescent protein at an MOI of 1,000 pfu/cell and visualized in a fluorescent microscope. One islet contains about 1,000 cells on average. Shown are murine islets that were mock-treated (A) and rAAV1-GFP transduced (B), as well as human islets that were mock-treated (C) and rAAV2-GFP transduced (D).

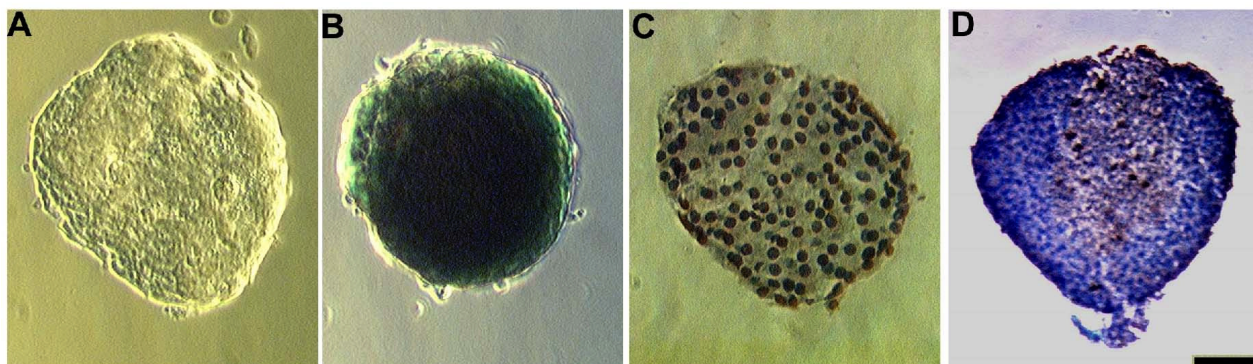


Figure 4. Lentiviral-mediated transduction of islets. Freshly isolated mouse islets were mock-transduced (A) and transduced with the FIV-LacZ vector at an MOI of 100 transducing units/cell (B) and stained for -gal after 24 h of incubation in the CMRL-1066 medium. In addition, after transduction with the FIV-LacZ vector, islets were paraffin-embedded and thin-sections of embedded islets were immuno-stained for insulin (C, brown) and stained with X-gal for -gal (D, blue). Bar, 25 μm.

d. Nonviral vector-mediated gene transfer to islets

In addition to viral-mediated gene delivery systems, nonviral systems such as liposome-mediated transfection have been used to deliver genes to a variety of cells both *in vitro* and *in vivo* (Ledley et al, 1995). Cationic liposomes are artificial membrane vesicles that can complex with DNA. The resulting liposome-DNA complex is thought to fuse with the negatively charged plasma membrane (Felgner and Ringold, 1991) or become endocytosed (Zhou and Huang, 1994), resulting in gene delivery to the nucleus.

It has been shown that islet cells in a monolayer derived from dispersed islets or intact islets can be effectively transduced using the monoliposomal reagent

Lipofectin or the polycationic liposome Lipofectamine or adenovirus-polylysine (AdpL) DNA complexes (Welsh et al, 1990; Welsh and Andersson, 1994; Saldeen et al, 1996; Benhamou et al, 1997). Recently, Mahato and colleagues (Mahato et al, 2003) reported that human islets transduced with the hVEGF gene by nonviral-mediated gene transfer resulted in sustained hVEGF production for up to 10 days post transduction. Although nonpathogenic, nonviral-mediated gene transfer is in general associated with a relatively low efficiency and short duration of transgene expression (Lakey et al, 2001). It has been suggested that after liposome-mediated endocytosis, a vast majority of lipid-DNA particles are retained in the perinuclear area and subsequently degraded (Zabner et al, 1995). Thus, the

failure of DNA to leave the endosomal compartment represents a major hurdle to liposome-mediated gene transfer. Nonviral-mediated gene transfer systems are of a preferred choice when persistent transgene expression is not desirable.

Recently, a novel system, known as protein transduction, is being developed. Unlike gene transfer systems, this protein transduction system allows selective delivery of proteins into cells, when linked to a specific protein transduction domain (PTD). PTD is a small peptide domain that can freely cross the cytoplasmic membrane through a receptor-mediated process, which is independent of ATP (Hawiger et al, 1999; Schwarze et al, 2000). In particular, a PTD designated PTD-5, which is originally selected from an M13 phage peptide display library, has been reported to successfully transduce both human and mouse islets without significant effects on islet function (Mi et al, 2000; Rehman et al, 2003). Likewise, Embury et al, (2001) also showed that a small peptide of 11 amino acid residues that constitute the PTD of the HIV/TAT protein, when fused to β -galactosidase, is able to transduce rat islets *ex vivo* with the fusion protein in a dose-dependent manner at a relatively high efficiency. However, such a protein transduction system is normally associated with a transient effect, depending on the relative stability of the fusion protein. In addition, for therapeutic protein delivery, caution should be taken to ascertain that the fusion of a PTD does not adversely affect the proper folding and compromise the function of the therapeutic protein.

III. Conclusion

Rapid re-establishment of an appropriate microvascular system in newly transplanted islets is crucial for survival and function of islet grafts. Unfortunately, islets implanted at ectopic sites, such as under the renal capsule or in the liver and spleen, are invariably associated with markedly reduced vascularization, in comparison with native islets in the pancreas (Beger et al, 1998; Mattson et al, 2002). This impairment in islet revascularization accounts at least in part for the demand of sufficiently large quantities of islet mass for restoration of normoglycemia in type 1 diabetic subjects. In addition, delayed and inadequate islet graft vascularization can deprive islets of oxygen and nutrients, causing islet cells to undergo cellular apoptosis and subsequent cell death, particularly in the core of large islets or in the center of aggregated islet clusters post transplantation. Moreover, a lack of sufficient islet revascularization may also compromise the optimal performance of transplanted islets. Indeed, there are clinical data indicating that even after postabsorptive blood glucose homeostasis is restored to normal post islet transplantation, implanted islets do not seem to function at optimal levels, as reflected in their significantly impaired glucose tolerance in diabetic recipients in response to intravenous glucose challenge (Ryan et al, 2001, 2002). Thus, it is of great significance to define the molecular mechanism of islet revascularization and develop therapeutic angiogenesis approaches to enhance the process of islet revascularization. Such approaches are

expected to ensure adequate microvascular perfusion to islet cells and protect implanted islet cells from hypoxia-induced inflammation and necrosis, which will ultimately improve the outcome of islet transplantation by reducing the donor/recipient ratio thus increasing the success rate of islet transplantation.

Acknowledgement

We would like to thank Marcia Meseck for critical reading of this manuscript. This project is supported partly by the Juvenile Diabetes Research Center at Mount Sinai School of Medicine.

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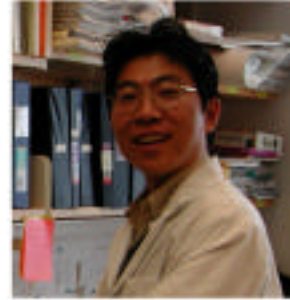
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