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Biological and Biomaterial Approaches for Improved Islet Transplantation

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***Abstract*——Islet transplantation may be used to treat type I diabetes. Despite tremendous progress in islet isolation, culture, and preservation, the clinical use of this modality of treatment is limited due to post-transplantation challenges to the islets such as the failure to revascularize and immune destruction of the islet graft. In addition, the need for lifelong strong immunosuppressing agents restricts the use of this option to a limited subset of patients, which is further restricted by the unmet need for large numbers of islets. Inadequate islet supply issues are being addressed by regeneration therapy and xenotransplantation. Various strategies are being tried to prevent β-cell death, including immunoisolation using semipermeable biocompatible polymeric capsules and induction of immune tolerance. Genetic modification of islets promises to complement all these strategies toward the success of islet transplantation. Furthermore, synergistic application of more than one strategy is required for improving the success of islet transplantation. This review will critically address various insights developed in each individual strategy and for multipronged approaches, which will be helpful in achieving better outcomes.**

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I. Introduction

Diabetes mellitus is a global disease with immense economic and social burden. type I diabetes is insulin-dependent diabetes mellitus (IDDM[1]) and is often called juvenile-onset diabetes. This autoimmune disorder leads to the destruction of insulin-producing pancreatic β-cells (Mathis et al., 2001). On the other hand, type II diabetes is noninsulin-dependent diabetes mellitus (NIDDM) and is often called adult-onset diabetes. Type II diabetes arises from peripheral resistance to insulin, leading to insulin overproduction by islets. As the disease progresses, the insulin-producing β-cells in the islets of Langerhans in pancreas get desensitized to the persistently high glucose signal, thus leading to reduced insulin production by islets in response to normal glycemic stimulation (Costa et al., 2002). Islet dysfunction plays a key role in late-stage type II diabetes (Porte and Kahn, 1995; Pratley and Weyer, 2001). Late-stage type II diabetic patients often require insulin therapy in much higher doses because of peripheral insulin resistance (Holman and Turner, 1995). The number of people with diabetes is expected to exceed 350 million by 2010, and 10% of these are expected to have type I diabetics (Serup et al., 2001). Although some of the approaches outlined in this review will also be relevant for treating type II diabetes, we will focus mainly on islet transplantation as a treatment option for type I diabetes.

Current approaches for treating type I diabetes include 1) exogenous insulin therapy and 2) pancreas transplantation. Although daily glucose monitoring and exogenous insulin administration has been the standard therapy since the discovery of insulin, the poor control of blood glucose fluctuations with this therapy leads to many severe complications including neuropathy, nephropathy, retinopathy, heart disease, and atherosclerosis (Bailes, 2002; Bloomgarden, 2004; Hill, 2004). In 1993, the Diabetes Control and Complications Trial showed that strict control of blood glucose levels reduced the risk of developing diabetes-related complications. Many improvements in the formulations and delivery systems of insulin promise to improve therapeutic outcomes of insulin administration. However, poor patient compliance, the inherent complications of using certain devices for insulin delivery, and the risk of hypoglycemia have prompted the search for a "cure" of diabetes. Pancreas transplantation is currently the only option available that promises to cure the disease. This procedure, however, requires major surgery and lifelong immunosuppression (Robertson et al., 2000). Therefore, most pancreas transplantations are done in diabetic patients with severe late-stage complications and undergoing kidney transplantation and immunosuppression. These procedures are known as simultaneous pancreas kidney or pancreas after kidney transplantation (Sutherland et

[1] Abbreviations: IDDM, insulin-dependent diabetes mellitus; NIDDM, noninsulin-dependent diabetes mellitus; VEGF, vascular endothelial growth factor; MHC, major histocompatibility complex; APC, antigen-presenting cell; TCR, T-cell receptor; IE, islet equivalent(s); FasL, Fas ligand; ICAM intercellular adhesion molecule; NO, nitric oxide; TNF, tumor necrosis factor; IL, interleukin; IFN, interferon; ALS, antilymphocyte serum; FTY720, 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride; TGF, transforming growth factor; Th, helper T-cell; Adv, adenovirus/adenoviral; Treg, $CD4^+CD25^+$ regulatory T-cells; huPBL, human peripheral blood leukocyte; TNFi, inhibitor of tumor necrosis factor; CTLA-4 cytotoxic T-lymphocyte antigen-4; CTLA-4-Ig CTLA-4 fused with immunoglobulin G1 Fc region; LFA-1, leukocyte function antigen-1; PERV, porcine endogenous retrovirus; Cre-ER, Cre protein fused with estrogen receptor; RIP, rat insulin promotor; HPAP, human placental alkaline phosphatase; CTV, cytomegalovirus; HGF, hepatocyte growth factor; PDX-1, pancreatic and duodenal homeobox gene 1; ES, embryonic stem; HEMA, 2-hydroxyethylmethacrylate; PLL, poly(L-lysine); PVP, poly(vinyl pyrrolidone); PEG, poly(ethylene glycol); NPCC, neonatal porcine cell cluster; GLP-1, glucagon-like peptide-1; VAPG, [poly(*N*-vinylpyrrolidone)-coacrylic acid-g-PEG-GLP-1]; EGFP, enhanced green fluorescent protein; hVEGF, human VEGF; RCA, replication competent adenoviruses; MOI, multiplicity of infection; RGD, arginine-glutamate-aspartate; iNOS, inducible NO synthase; ODN, oligonucleotide; RNAi, RNA interference; siRNA, small interfering RNA; G6PD, glucose-6-phosphate dehydrogenase.

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al., 2001). Pancreas transplantation is, therefore, not available to a vast majority of diabetic patients as a therapeutic option.

Islet transplantation, on the other hand, promises to be a cure at least as effective as pancreas transplantation, while being much less invasive. Islet transplantation involves the isolation of functional islets from cadaveric, multiorgan donors. These islets are then injected into the hepatic portal vein of the diabetic patient, from where they get deposited in well-perfused liver sinuses. Islet transplantation differs from other tissue and organ transplantation approaches in being a heterotropic graft, a graft that is located on a site other than the natural location of the tissue (Rossini et al., 1999). Islets are not transplanted homotropically in the pancreas of the recipient because the pancreas is a highly sensitive tissue. Any injury or manipulation of the pancreas leads to severe pancreatitis, with accompanying pain and tissue destruction (Morrow et al., 1984). Islet transplantation can provide certain advantages that are not available with pancreas transplantation including the potential for modifying tissue immunogenicity through in vitro culture or gene therapy approaches, tissue encapsulation for immunoisolation, potential for engraftment in immunoprivileged sites, and the possibility of using alternative tissue sources including xenogenic islets and stem cell derived β-cell lines.

Scientists have made many advances in islet transplantation in recent years. In June 2000, Shapiro et al. at the University of Alberta in Edmonton, Canada, published results of an exceptionally successful case of islet transplantation wherein seven of seven patients were insulin-free at the end of 1 year (Shapiro et al., 2000). The latest results of their phase II clinical trials indicate a success rate of 82% with islet transplantation alone carried out in type I "brittle" diabetic patients (Oberholzer et al., 2003). This success rate matches that of pancreas transplantation (Sutherland et al., 2001) and has essentially revitalized this field and led many new centers to initiate the program of islet transplantation (Chang et al., 2004). Widespread clinical application of this procedure, however, is currently limited by the need for lifelong immunosuppression and the need for two to four donor pancreases per recipient.

The challenges to successful transplantation of islets include 1) isolation, culture, characterization, and preservation of islets, 2) inflammation and autoimmune-mediated destruction and alloimmune rejection of transplanted islets, 3) failure to revascularize, 4) low transplanted mass and high metabolic demand on the tissue, and 5) a limited supply of islets for widespread clinical use (Table 1) (Robertson, 2001; Lakey et al., 2003; Ricordi and Strom, 2004). The major barrier to islet transplantation is the need for lifelong immunosuppression of the recipient. Once this barrier is overcome, the limitation of islet supply will hamper the use of this procedure (Larkin, 2004). In the present review, we discuss these current challenges to the success of islet transplantation and the application of biological and biomaterial-based approaches toward improving the clinical success of islet transplantation.

Prevention of immune rejection of transplanted islets has conventionally been the use of generalized immunosuppression with its significant side effects (Inverardi et al., 2003; Chang et al., 2004). Several immune-modulation strategies have recently evolved to selectively block the immune responses against the graft. Some of these strategies are already used clinically for other applications, e.g., muromonab-CD3 in acute renal rejection (Smith, 1996), whereas others have been tested individually or in combination in several animal models. These will be discussed in section III.A.

To generate alternative sources of islets, both the use of islets from alternative species (xenotransplantation) and generation of islets and/or insulin-producing cells of human origin (regeneration therapy) have been explored (Scharfmann, 2003; Hussain and Theise, 2004). Whereas the former source suffers primarily from enhanced immune destruction of transplanted tissue due to xenoantigen rejection, the latter has met with limited success hitherto in generating large amounts of tissue. The use of replicating cell lines, on the other hand, has significant safety concerns for human applications. These issues and progress in these fields are discussed in section III.B.

Biomaterials have the potential to improve the outcome of islet engraftment by encapsulating islets before transplantation. This strategy of immunoisolating islets has met with limited success due to engineering, pro-

TABLE 1
Reasons for islet graft failure and useful interventions

Obstacles to Islet Transplantation	Strategies
Loss of viability during isolation	Improved enzymatic blends for islet isolation, culture media composition, and conditions
Failure to revascularize	Encapsulation with vascular growth promoting proteins; ex vivo growth factor gene delivery for revascularization
Inflammatory response/immune rejection	Immunoisolation membranes
	Generalized immunosuppression
	Immune tolerance induction
	Antiapoptotic and immune modulating gene delivery
Inadequate islet mass	Optimization of islet mass and transplantation site
Inadequate islet supply	Xenotransplantation
	Stem cell-based approaches

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cess, and biomaterial limitations. Furthermore, the diffusional barrier limits the free supply of oxygen and nutrients, resulting in hypoxia and lack of revascularization of islets. These problems may be circumvented by using surface coating of islets. These approaches are elaborated on in section IV.

An alternative to physical isolation of the transplanted tissues from the host involves modulating the gene expression profile of islets before transplantation by nucleic acid-based approaches. These strategies include both gene expression and gene knockdown approaches through the use of either nonviral or viral vectors. Furthermore, ex vivo transfection of islets before transplantation has the potential to be safely included in the clinical islet transplantation protocol. Several promising strategies will be discussed in section V. We finally conclude with an outlook for the future and the strategies that hold the most promise for solving some of the toughest problems currently hampering the clinical success of islet transplantation.

II. Obstacles to the Success of Islet Transplantation

Islet transplantation may be done in three different modalities (Federlin et al., 2001). Transplantation of islets isolated from the same animal is referred to as autologous or autotransplantation, transplantation of islets from the same species is referred to as allogeneic or allotransplantation, and xenogenic or xenotransplantation refers to the use of islets from a different species. Syngeneic transplantation is a special case of allotransplantation in which the graft donor and the recipient are monozygotic. Autotransplantation involves transplanting islets of the patient to himself or herself in patients who necessitate total pancreatectomy, e.g., chronic pancreatitis (White et al., 2000). Although allotransplantation is the preferred modality for immunological and safety reasons, severe constraints in tissue availability necessitate exploring getting islets from alternative sources (xenotransplantation) to make this option available to a substantial fraction of patients.

In the overall process of islet transplantation, islets are obtained from the pancreas of cadaveric multiorgan donors in the case of allotransplantation or from animals (e.g., pig) in the case of xenotransplantation. The pancreases are digested with collagenase that disintegrates the intercellular matrix of collagen, thus releasing islets. The islets are isolated, purified, tested, and cultured before being transplanted into the recipient, which is usually done by a simple injection in the hepatic portal vein that deposits the islets in the liver. The overall process of islet isolation, purification, preservation, and quality control poses serious challenges to the clinical outcome of islet transplants (Menger and Messmer, 1992; London et al., 1994; Lakey et al., 2002).

A. Loss of Islet Viability during Isolation and Culture

Efficient isolation of pure islets, without inflicting significant damage, is the key to successful islet transplantation. The pancreas contains exocrine, endocrine, and ductal cells. The endocrine cells, arranged in islets within the pancreas, consist of α-, β-, δ-, and PP cells that secrete glucagon, insulin, somatostatin, and pancreatic polypeptide, respectively (Menger et al., 1994). Figure 1 shows the location of the pancreas inside the abdominal cavity and the microanatomy of islets. Islet portal circulation, with blood flow from β- to α- to δ-cells, as well as afferent innervation from the central nervous system, have a role in hormone secretion from constituent cells (Helman et al., 1982; Steffens and Strubbe, 1983). Islet isolation from the pancreas essentially involves dissociation of islets from the exocrine pancreas by enzymatic digestion combined with mechanical agitation. Isolated islets are then purified by density gradient centrifugation. A critical balance of composition, process, and duration of collagenase digestion is required for isolating islets with high purity, integrity, and viability with a sufficient yield. The enzymatic digestion process disrupts islet-to-exocrine tissue adhesive contacts (Wolters et al., 1992). Thus, whereas lower duration or inappropriate composition of collagenase will lead to incomplete purification of islets from exocrine tissue along with reduced yield, increased duration of collagenase exposure adversely affects within-islet cell-to-cell adhesion, leading to loss of islet integrity and viability.

The associations of cells within the islets and those between the islets and the exocrine pancreatic tissue may be either cell-to-cell or cell-to-matrix. The nature of these associations was investigated by Van Deijnen et al. (1992) in rat, dog, pig, and human pancreas. The authors found that intraislet associations are predominantly cell-to-cell in all four species, whereas islet-to-exocrine tissue interactions are predominantly cell-to-cell in pig pancreas and cell-to-matrix in canine pancreas, which has completely encapsulated islets. In the case of rat and human pancreas, the situation was intermediate with a tendency toward predominance of cell-to-matrix interactions. These observations explain the reason that pig islet isolation is difficult (White et al., 1999; Omer et al., 2003) and also point to the kind of digestive enzyme mixtures that may be used for a given species. For example, intraislet cell-to-cell adhesion is protease-sensitive, whereas the extraislet cell-to-matrix adhesion is collagenase-sensitive (McShane et al., 1989; Wolters et al., 1992). Thus, the use of highly pure collagenase preparations is desirable to isolate pure islets with the least possible damage to the islets themselves. The presence of protease in the collagenase preparations reduces the yield and quality of isolated islets (Vos-Scheperkeuter et al., 1997). However, removing exocrine tissue is more efficient with the use of collagenase prep-

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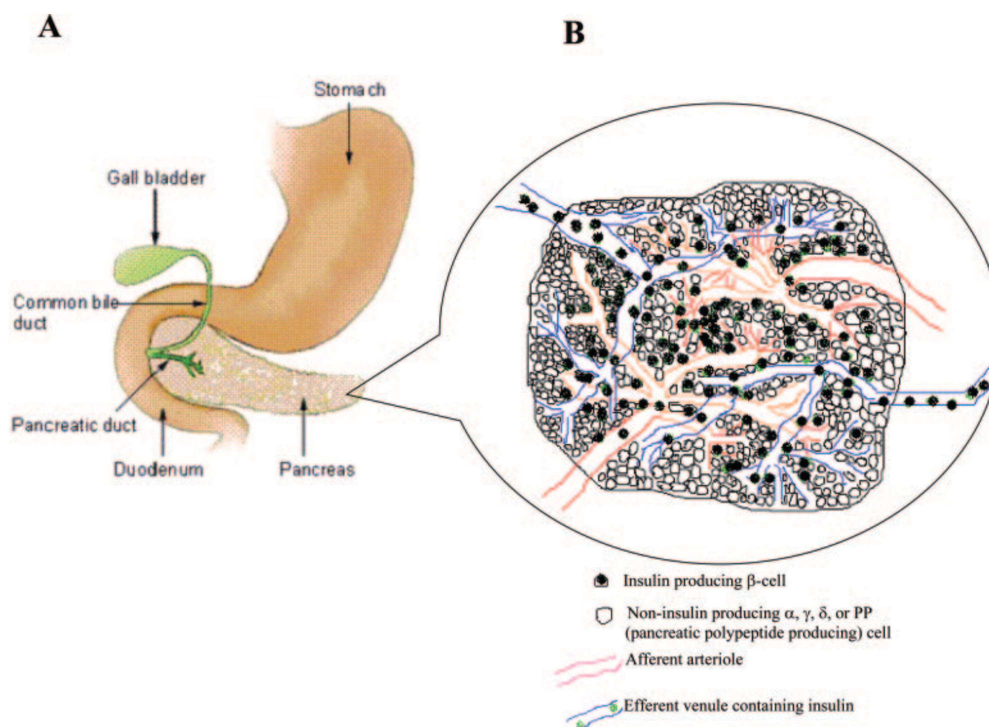

FIG. 1. Location of the pancreas inside the abdominal cavity (A) and the microanatomy of constituent endocrine islets distributed throughout the pancreas (B).

arations containing protease for the isolation of pig islets (van Deijnen et al., 1992). Different enzyme composition and process modifications have been evaluated for isolation of pancreatic islets (Lakey et al., 1998b; Bucher et al., 2005). These include the use of additives in the collagenase solution (Arita et al., 2001), composition of density gradient materials (Lakey et al., 1998a), and digestion procedures (Lakey et al., 1999). Such studies have led to development of various species-specific liberase enzyme blends (Linetsky et al., 1997; Brandhorst et al., 1999; Olack et al., 1999) and the automated Ricordi chamber (Poo and Ricordi, 2004) for optimized islet isolations. These aspects, however, are not discussed in detail here, and the interested reader is referred to these publications.

The use of slightly impure islet preparations and coculture with extracellular matrix components such as collagen (Nagata et al., 2002) have been shown to enhance the viability and function of isolated islets. In addition, supplementation of culture medium with small intestinal submucosa was shown to improve islet functioning and viability (Lakey et al., 2001a). Media composition, seeding density, and temperature play a significant role (Falqui et al., 1991; Murdoch et al., 2004). In addition, islet coculture with pancreatic ductal epithelial cells was shown to be useful for maintaining islet viability and function after isolation (Gatto et al., 2003). Pancreatic ductal epithelial cells have been considered as putative stem cells for islets and an essential component of the extracellular matrix, which plays an important role in secreting appropriate growth factors that support islet viability. Gatto et al. (2003) found that long-term culture, as well as cryopreservation, decreased the viability of human pancreatic islets, which was prevented by coculture with ductal epithelial cells at 33°C. In a different study, coculture with ductal epithelial cells helped maintain structural integrity and viability of the islets (Ilieva et al., 1999).

Fraga et al. (1998) have evaluated different media supplements for the extended culture of human pancreatic islets. Islet culture in Connaught Medical Research Laboratories medium (Life Technologies, Inc., Rockville, MD) was compared with the supplementation of either 10% fetal bovine serum (standard medium) or insulin-, transferrin-, and selenium-containing medium (also known as the Memphis medium). Long-term culture of islets in insulin-, transferrin-, and selenium-containing medium was shown to maintain the viability of islets with no adverse effect on in vivo function in the NOD-SCID mouse model (Gaber et al., 2001; Gaber and Fraga, 2004; Rush et al., 2004) and correlated with islet function after transplantation in human subjects (Gaber et al., 2004).

Islet viability during culture is also adversely affected by hypoxia to the cells in the inner core of islets (Dionne et al., 1993; Gorden et al., 1997; Vasir et al., 1998). Although it may be difficult to prevent a hypoxic condition of the inner islet cell mass during in vitro culture, genetic modulation of islets to express genes that promote rapid revascularization upon transplantation and reduced culture time could play an important role in preventing hypoxic damage to the islets (Mahato et al.,

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2003; Cheng et al., 2004; Narang et al., 2004). These approaches are discussed in section V.

B. Inadequate Revascularization of Transplanted Islets

Islets are like an organ in themselves with extensive intraislet vasculature, formed of fenestrated capillary endothelial cell lining, which is essential for the supply of oxygen and nutrients to the cells in their inner core (Carroll, 1992; Menger et al., 1994). To determine the presence and orientation of intraislet vasculature, Menger et al. (1992) transplanted 8 to 10 isolated hamster islets into the dorsal skinfold chamber of syngeneic animals. Fourteen days post-transplantation, the microvasculature of the transplanted islets was perfused by an injection of 200 μl of 5% fluorescein isothiocyanate-conjugated dextran (mol. wt. 150,000), and the islet vasculature was analyzed by intravital fluorescence microscopy. As seen in Fig. 2, the supporting arterioles penetrate into the periphery of the islet and break into capillaries within the graft. Glomerulus-like capillary perfusion is directed to microvessels located within the core of the islet (Menger et al., 1994).

Islets are so well perfused in vivo that they receive 5 to 15% of their total blood flow of the pancreas even though they constitute ~1% of the pancreas by weight (Lifson et al., 1980; Jansson and Hellerstrom, 1983). This vasculature gets disrupted during the process of islet isolation and culture, which causes an accumulation of endothelial fragments and compromises perfusion of the core of islets. Therefore, rapid revascularization is crucial for islet engraftment, survival, and function post-transplantation (Brissova et al., 2004). Successful islet grafts have been observed to regenerate the microvasculature within 10 to 14 days of transplantation (Menger et al., 1994; Vajkoczy et al., 1995; Merchant et al., 1997; Beger et al., 1998; Furuya et al., 2003). However, the proportion of islets that restore their original vasculature upon transplantation is limited and variable. This issue is as a fundamental factor in determining long-term graft survival and function.

Because of the disruption of intraislet vasculature, islets in culture, as well as during the initial few days of transplantation, depend on the diffusion of oxygen and nutrients from the periphery. Vascular endothelial cells are lost during culture (Mattsson, 2005), making endothelial cell expansion essential to the islet revascularization process. Islet survival and long-term function after transplantation are often antagonized by the lack of reestablishment of capillary networks within the islets (Narang et al., 2004), which also exacerbates immune destruction of transplanted islets (Lukinius et al., 1995).

Although transplantation in highly perfused organs such as the liver promises to provide adequate tissue bathing to provide nutrition by diffusion, the cells in the inner core of the islets still do not receive an adequate supply of oxygen and nutrients. These cells depend on intraislet capillary-mediated flow of blood. This limitation leads to lower oxygen and nutrient supply in the inner core of islets, which constitutes predominantly the insulin-secreting β-cells, and ultimately leads to hypoxia and cell death. This phenomenon was elegantly demonstrated by Vasir et al. (1998), who stained islets cultured for 24 and 48 h with propidium iodide (red color) and calcein-AM (green color) to demonstrate the progressive loss of islet viability in the center of the islets. This loss

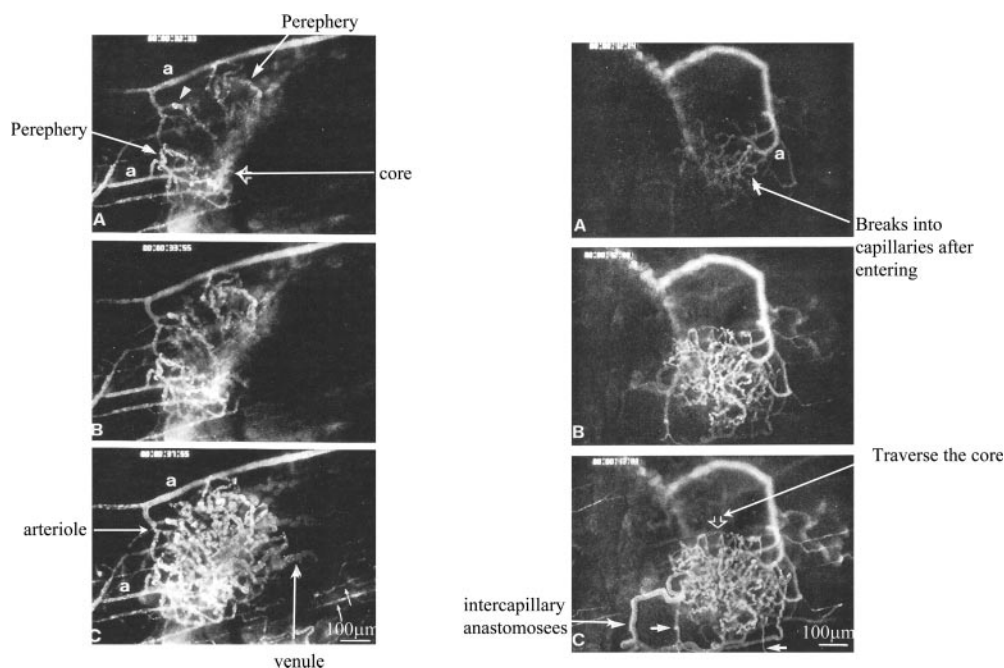

FIG. 2. Experimental demonstration of intricate intraislet vasculature by i.v. administration of dextran-fluorescein isothiocyanate and fluorescence imaging. Reproduced from Menger et al. (1994) and republished with permission.

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of viability was associated with hypoxia in the inner core cells of islets. Culturing the same islets for an additional 24 h under hypoxic conditions exacerbated cell death (Vasir et al., 1998).

Islets try to revascularize themselves by secreting pro-angiogenesis molecular mediators such as vascular endothelial growth factor (VEGF) and its receptors (Vasir et al., 2000. 2001). Revascularization of islets post-transplantation occurs from the surrounding host tissue vasculature (Konstantinova and Lammert, 2004; Narang et al., 2004; Zhang et al., 2004). Secretion of VEGF and similar pro-angiogenic factors by the islets tends to promote this process. Achievement of rapid revascularization is expected to improve the viability and functioning of transplanted islets. This may be achieved by ex vivo *VEGF* gene delivery to islets (Narang et al., 2004) or by coencapsulating VEGF protein with islets during microencapsulation (Sigrist et al., 2003a). These approaches are discussed in section V.

C. Autoimmune Recurrence and Immune Rejection

Immunological challenges to islet survival, engraftment, and function post-transplantation are 2-fold: alloimmune destruction and autoimmune rejection. Although the former is common to all organ and tissue transplantation situations, type I diabetes offers additional challenges because it is autoimmune in origin. Diabetes is characterized by the presence of β-cell-reactive autoantibodies and T-cells in the patient. Although the β-cell lesion is mediated by β-cell-specific autoreactive T-cells, the specific nature of effector T-cells remains elusive. The host has pre-existing antibodies and primed immune cells against β-cell surface epitopes and insulin, which participate in graft destruction, in addition to the immune cells that infiltrate in response to nonself antigens (Jaeger et al., 2000). The host also reacts to nonself proteins originating from the transplanted tissue in the case of allo- and xenotransplantation. In terms of nonself antigens, immunological closeness of the graft to the host significantly influences the magnitude of an immune attack and determines overall graft survival outcome. Hence, success rate of transplantation is in the following order: autotransplantation > allotransplantation > xenotransplantation.

An understanding of the mechanisms underlying host immune responses in the context of islet transplantation is useful to the application of various immunosuppressive, immune-modulating, and immune-tolerizing approaches to improving transplantation outcome. The immunological bases of islet rejection overlap with transplant rejection situations involving heart, lung, liver, pancreas, and kidney transplantation such that the knowledge generated by research in these areas can be applied to islet transplantation. Although the overall basics of immunology and its application to the allograft situation are reviewed elsewhere (Rossini et al., 1999; Janeway et al., 2001), we will focus on the strategies that have been applied to islet transplantation and the underlying immune processes that are modulated.

When foreign tissue is transplanted, the host recognizes foreign antigens. This recognition follows the process of "antigen presentation" to the host immune cells, whereby peptide fragments of various surface, secreted, and shed proteins are brought in direct contact with the host immune cells attached to glycoproteins known as the major histocompatibility complex (MHC). The MHC can be class I or class II, depending on the cells that express these complexes. Whereas MHC class I molecules are expressed on all cells of the body, MHC class II molecules are expressed only on the surface of certain immune cells including macrophages and dendritic cells, which are known as professional antigen-presenting cells (APCs). Both class I and class II MHCs are able to present foreign peptides to host immune cells, but only class II MHC-bound peptides elicit an immune response because of the "costimulation" requirement of host T-cells for their full activation (Rossini et al., 1999).

Foreign antigen presentation to the host can be done by the host's own APCs or those of donor origin. The host APCs may be the mononuclear cells that infiltrate the graft and migrate away from the graft, or they may be the circulating APCs that encounter soluble donor antigens that have diffused away from the graft. Such soluble donor antigens are present predominantly in the case of xenografts. Donor APCs, on the other hand, are usually the dendritic cells, macrophages, and the circulating T-lymphocytes (called passenger leukocytes) that are transferred along with the graft. The antigens carried by the MHC II molecules on these APCs act as foreign antigens to the transplant recipient when these lymphocytes migrate to the lymph nodes of the host.

Donor antigen presentation to the host T-cells could be mediated via a direct or indirect pathway. The direct pathway involves antigen presentation by the donor APCs, whereas the indirect pathway involves host APCs, which pick up and process donor antigens for presentation as illustrated in Fig. 3. In the direct pathway of immune destruction, predominant in the case of allotransplantation, donor APCs migrate from the implantation site and present antigens to the host T-cells resulting in the development of $CD4^+$ helper Th1 cells (Nicolls et al., 2001; Jiang et al., 2004). Th1 cells, in turn, produce a set of cytokines that favor expansion and activation of cytotoxic $CD8^+$ T-cells. These are the primary effector cells mediating allogeneic cell damage. Xenotransplantation of islets, however, leads to the activation of the indirect pathway whereby antigens shed by the donor are taken up by host APCs and displayed on MHC II molecules (Watschinger, 1995; Game and Lechler, 2002; Jiang et al., 2004). In the case of xenotransplantation, humoral responses targeted mainly toward the surface α-(1,3)-galactose moiety already exist. This moiety is present in animal tissue but has been evolutionarily lost in humans. The presence of these

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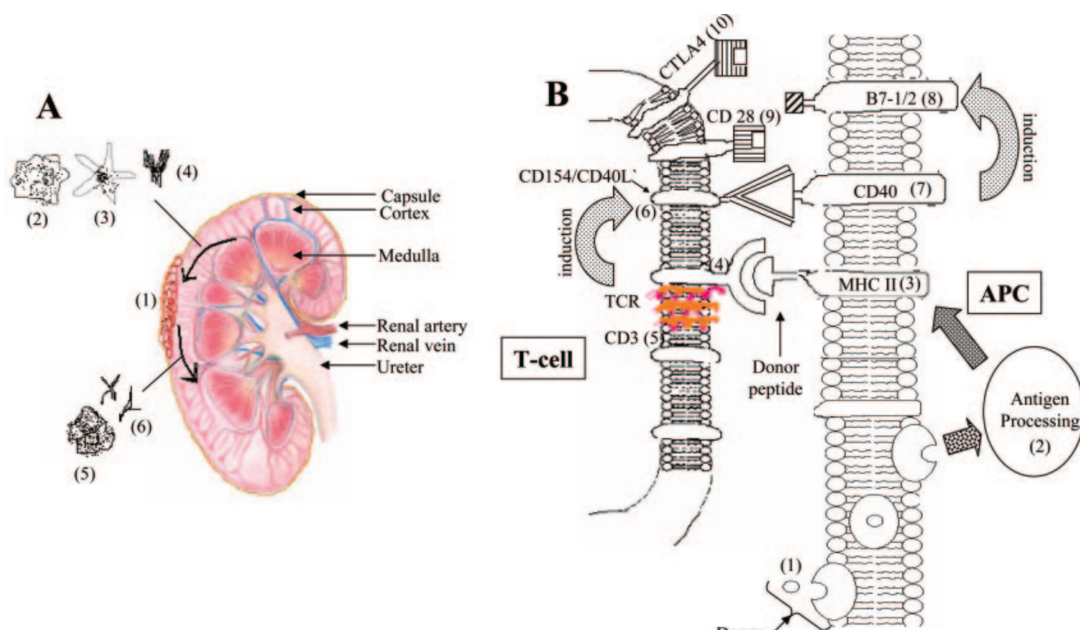

FIG. 3. Host immune response to islets engrafted under the kidney capsule. A, islets transplanted under the kidney capsule (1) being infiltrated by host macrophages (2), dendritic cells (3), and antibodies (4). The infiltrating host APCs present graft antigenic peptides to host T-cells (indirect antigen presentation pathway). The islet graft releases passenger leukocytes (5) and antigens (6) into the circulation. These directly present antigens to the host T-cells (direct antigen-presentation pathway). Activation of host T-cells by professional APCs is illustrated in B. Professional APCs pick from the graft site (1) donor islet antigens, which are then processed (2), and the antigenic peptides are presented on the MHC class II molecules (3) to the host TCRs (4), which exist in close proximity to constitutively expressed three-transmembrane domain CD3 molecules on the T-cell surface (5). Upon TCR activation, host T-cells express CD40 ligand or CD154 (6), which binds with constitutively expressed CD40 on the APCs (7). This interaction stimulates the APCs to up-regulate cell surface expression of B7-1/2 and T-cells to transiently express CTLA4. Interaction of B7-1/2 (8) with constitutively expressed CD28 (9) and/or transiently expressed CTLA4 (10) on T-cells provides a second costimulatory signal. These costimulation signals are critical to the full activation of host T-cell response. For this reason, these interacting cell surface molecules are also targets of many immune tolerance strategies.

preformed antibodies is the main cause of hyperacute rejection observed with xenotransplantation (Cozzi et al., 2000; Ramsland et al., 2003). Although donor APCs are present in the transplanted xenogenic islets also, they are not able to activate the direct pathway because the MHCs displayed on donor APCs are not able to efficiently engage host T-cells (Chitilian et al., 1998; Vallee et al., 1998).

An understanding of these pathways is critical to the design of approaches for preventing immune destruction of transplanted islets. Although allogeneic islet destruction involves predominantly cytokine action and cytotoxic $CD8^+$ T-cells, xenogenic tissue destruction proceeds via the antigen-antibody reaction pathway (Makhlouf et al., 2003). Thus, preventing leaching of cellular antigens is of critical significance to the biomaterial-based approaches to xenotransplantation. In contrast, preventing the migration of donor APCs from the transplant site could be adequate for allotransplantation. These criteria have significant implications in terms of the porosity and diffusibility requirements for encapsulated systems containing allogeneic or xenogenic islets, the latter being more stringent. Hence, whereas ultrafiltration (2–50 nm pore size) membranes are required for xenograft applications, microfiltration (0.1–1 μm pore size) membranes are adequate for allotransplantation of islets (Chaikof, 1999).

In both pathways, the molecular level mechanism of T-cell activation involves the following five steps (Fig. 3): 1) recognition of MHC and the bound peptide by the T-cell receptor (TCR) on the host T-lymphocyte, which, together with intimately associated CD45 transduces Signal 1, 2) transient CD40L (also called CD154) expression on the responding T-cell, 3) interaction of CD154 (CD40L) with the constitutively expressed CD40 on the APC (Coactivation), 4) up-regulation of costimulatory molecules B7-1 and B7-2 on the APC membranes, and 5) interaction of B7-1/2 with constitutively expressed CD28 or transiently expressed cytotoxic T-lymphocyte antigen-4 (CTLA-4) on the T-cell (Costimulation, Signal 2). In the absence of costimulation, the host T-lymphocytes are not activated by contact with foreign antigen and may, in fact, undergo apoptosis. Various immune tolerizing strategies attempt to block immune responses at one or more of these steps. These are discussed in section III.A.

D. Islet Mass and Site of Transplantation

The total number of islets present in an adult human pancreas is approximately 1 million, however, only

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about one half or fewer of these are successfully isolated. Thus, whereas transplantation of one intact pancreas is adequate to achieve glucose homeostasis in a diabetic recipient, islet transplantation requires the use of islets from two to four donor pancreases. Therefore, >10,000 IE/kg were transplanted in diabetic patients using the Edmonton protocol (Street et al., 2004a), whereas Gaber et al. (2004) used 11,000 to 15,000 IE/kg over three different infusions. The islet mass requirement for transplantation is reflected not only in the achievement and maintenance of normoglycemia in transplant recipients, but also in terms of long-term graft survival and function (Rickels et al., 2005). Often transplanted islets do not engraft well, leading to primary nonfunction. Primary nonfunction occurs because of nonspecific events that are not related to the classic immune rejection phenomena. It is caused by the poor quality of islet preparation, cytokine-mediated local inflammation and apoptosis, blood clotting, and hypoxia before revascularization of the islets (Bretzel, 2003). Islets further experience high metabolic demand in the recipient because of insulin resistance, diabetogenic and toxic immunosuppressive agents (glucocorticoids, cyclosporine A, and tacrolimus), and low transplanted islet mass. If and when inadequate numbers of islets are transplanted, the increased metabolic demand and persistent hyperglycemia may lead to graft destruction from islet apoptosis (Rossetti et al., 1990; Leahy et al., 1992).

The number of transplanted islets plays a critical role in short- and long-term islet function and metabolic normalization in the transplant recipient (Beattie and Hayek, 1993; Tobin et al., 1993). Various researchers have investigated the effect of number of islets transplanted on various aspects of islet function post-transplantation. For example, Finegood et al. (1992) evaluated the time required for normalization of fed-state plasma glucose levels during the 5 weeks after syngeneic transplantation of 500 to 3000 IE in streptozotocin-induced diabetic Wistar Furth rats by portal vein infusion. Islet mass had an inverse correlation to the time to glycemic normalization (Fig. 4A). Animals receiving 500 IE required approximately 5 weeks to achieve normoglycemia, whereas animals receiving 2000 to 3000 IE achieved normoglycemia within 2 weeks of transplantation. Bell et al. (1994) observed that the blood glucose levels were inversely proportional to the islet mass. Increasing islet masses improves both short-term and long-term glycemic normalization and leads to prolonged graft survival, due to reduced hyperglycemic stress to the islets (Fig. 4B). In the xenotransplantation of human islets in the subcapsular space of NOD-SCID mice, Rush et al. (2004) demonstrated improvement in insulin production upon transplantation of a higher number of islets. They concluded that 2000 IE/mouse are adequate for in vivo assessment of islet function.

Another significant aspect of the number of islets used for transplantation is the glucotoxicity to β-cells. Islets release insulin in a typical biphasic manner upon an increase in glucose concentration (Bratanova-Tochkova et al., 2002; Kennedy et al., 2002; Straub and Sharp, 2002). Persistently increased glucose concentrations, however, are toxic to the islets (Kaneto et al., 1999; Francini et al., 2001; Leibowitz et al., 2001; Biarnes et al., 2002; Maedler et al., 2002b). Thus, rapid and effective normalization of blood glucose after islet transplantation—from either sufficient mass of islets, secretion of the required amount of insulin, or exogenous insulin administration—is critical to the survival and function

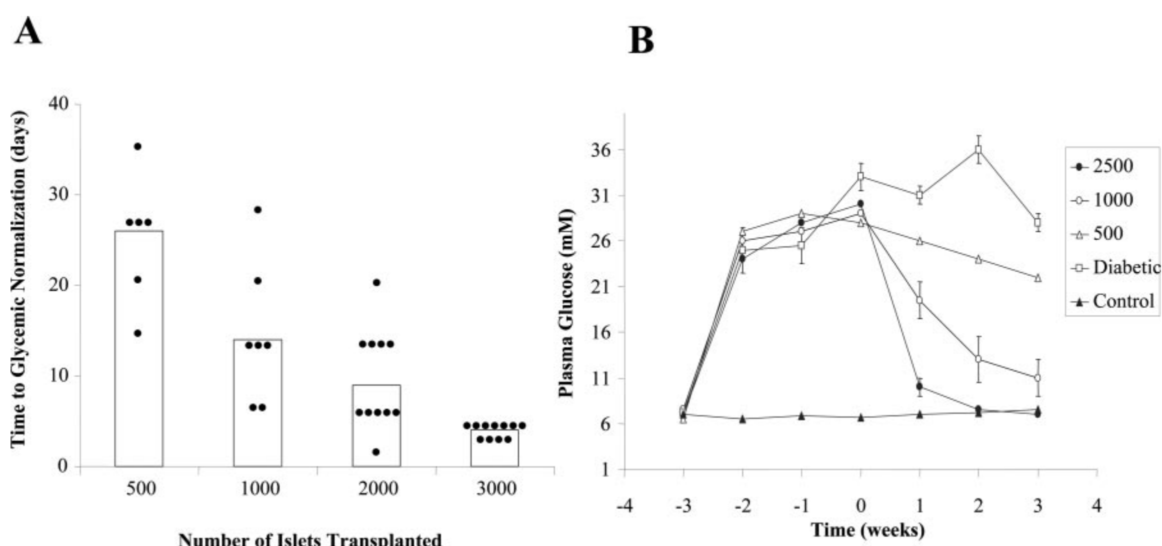

FIG. 4. Effect of islet mass on short-term and long-term glycemic normalization. A, time to glycemic normalization after transplantation. B, glycemic levels up to 3 weeks post-transplantation. A graded number of islets were used for allotransplantation in streptozotocin-induced diabetic rats in both studies. A was reproduced from "Dynamics of Glycemic Normalization Following Transplantation of Incremental Islet Masses in Streptozotocin-Diabetic Rats" [Finegood et al. (1992) *Transplantation* **53:**1033–1037]. B was redrawn from Bell et al. (1994) with permission; copyright © 1994, The Endocrine Society.

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of islet graft. Thus, Biarnes et al. (2002) found that increased islet apoptosis and increased islet mass upon suboptimal syngeneic islet transplantation (100 IE/mouse) in streptozotocin-induced diabetic mice under a chronic (30 days) hyperglycemic state compared with mice in which normoglycemia was maintained by exogenous insulin administration. Exogenous insulin administration is a standard part of the protocol of islet transplantation, and the insulin amount required is slowly tapered off over a period of time after the islets are transplanted.

The site of transplantation of islets also influences graft performance (al-Abdullah et al., 1995; Mahmoud et al., 1998). The various sites that have been evaluated for islet transplantation include the liver (Contreras et al., 2004a), spleen (Weitgasser et al., 1996), abdominal cavity (in the omentum) (Kin et al., 2003), testes (Gores et al., 2003), and renal subcapsular space (Molano et al., 2003). The rationale for selecting different sites in experimental systems often depends on a host of factors. For example, abdominal implantation is preferred for micro- and macroencapsulated systems because of higher volume of the graft. Transplantation in immunoprivileged sites such as the testes and under the kidney capsule have been preferred to reduce immunological challenges to the engrafted tissue (Ksander and Streilein, 1994). In fact, Sertoli cells, which contribute to the immunoprivileged status of the testes, have been cotransplanted with islets under the kidney capsule in an attempt to prolong islet allograft survival without systemic immunosuppression (Selawry and Cameron, 1993; Kin et al., 2002).

Immune privilege at these sites has been correlated to several factors, including high levels of Fas ligand (FasL) expression on cells (Bellgrau et al., 1995; Griffith and Ferguson, 1997). FasL-expressing cells interact with Fas-expressing T-lymphocytes in the graft region and lead to apoptosis of the infiltrating cells by the natural Fas-FasL-mediated process. This knowledge has led to attempts to ectopically express FasL in the islets or at the graft site to prevent acute graft rejection. Thus, Lau et al. (1996) genetically engineered myoblasts from the donor to express FasL. Islets from mice were allotransplanted with syngeneic myoblasts under the kidney capsule of streptozotocin-induced diabetic mice, leading to long-term normoglycemia. Transplantation of myofibroblasts expressing FasL on the other kidney led to graft failure, indicating the need for local FasL expression (Lau et al., 1996). The expression of FasL on islets themselves, however, met with failure, probably because the expressing islets brought infiltrating T-lymphocytes in closer proximity, which led to islet destruction (Kang et al., 1997). This result was also observed when FasL transgenic mice were prepared with β-cell promoter specific expression of FasL, such that it was expressed only on β-cells (Chervonsky et al., 1997). These studies indicate that FasL expression in proximity to the graft, but not from the graft itself, can prevent immune-mediated destruction of transplanted islets.

In a study directly comparing islet transplantation under the kidney capsule versus that under the spleen capsule, Weitgasser et al. observed more prolonged normoglycemia when syngeneic islets were transplanted under the kidney capsule in streptozotocin-induced diabetic rats (Weitgasser et al., 1996). The most widely used site for islet transplantation, however, is the liver, which follows portal vein administration of islets as a suspension (Shapiro et al., 2000). Transplantation in the liver is the least invasive. It ensures that each islet receives an ample amount of blood supply, and insulin production and utilization follow the physiological route (Arbit, 2004).

Islets transplanted in the hepatic portal vein lodge themselves in the sinusoids of the liver. Although intraportal transplantation has been used in most clinical studies, certain factors lead to significant islet damage and trauma. The benefit of ‘bathing in blood’ toward rapid diffusive transport of nutrients is offset by the adverse inflammatory reactions initiated by islets when they suddenly come in contact with blood in the portal vein. This instant blood-mediated inflammatory reaction of islets is characterized by the activation of coagulation and complement systems, islet infiltration of host leukocytes, and binding of host platelets (Bennet et al., 1999; Badet et al., 2002; Moberg et al., 2003). Islet damage also occurs because of nonspecific activation and dysfunction of intrahepatic host endothelial cells. These endothelial cells, in response to islets lodging in hepatic microcapillaries, up-regulate intercellular adhesion molecule (ICAM)-1 and P-selectin and produce nitric oxide (NO) and inflammatory cytokines such as TNF-α, IL-1β, and IFN-γ (Xenos et al., 1994; Bottino et al., 1998; Contreras et al., 2004a). Resident islet macrophages, Kupffer cells of the liver, and liver sinusoidal endothelial cells have been implicated as primary mediators of inflammation-mediated loss of islets when transplanted in the liver (Barshes et al., 2005). Furthermore, portal islet transplantation leads to bleeding, portal venous thrombosis, and portal hypertension (Robertson, 2004). These complications are partly offset by portal blood pressure monitoring and the use of anticoagulants during the procedure (Robertson, 2004).

Islet mass required for glycemic normalization is also influenced by the site of transplantation. For example, in the case of canine islet autografts, Kaufman et al. (1990) found that whereas the threshold number of islets required to achieve normoglycemia in the liver and spleen were similar (~4500 IE/kg), this number failed to ameliorate hyperglycemia when transplanted in the renal subcapsular space. Whereas both the islet mass and the site of islet transplantation in the host play an important role in graft survival and function, their definitive optimization is difficult because of a host of factors that influence ultimate graft survival. Although portal vein transplantation of islets at a count of >10,000 IE/kg recipient is contemporarily practiced,

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these variables may be optimized for further improvement with various interventions being explored to improve islet graft survival and function.

III. Biological Strategies for Improving the Success of Islet Transplantation

The two major impediments to the clinical success of islet transplantation are the immune destruction of transplanted islets and the limited supply of islet tissue. Several approaches have been proposed and tested to address these problems. Among the biological strategies used to overcome immune rejection are the use of novel immunosuppressive agents and regimens, and donor-specific induction of immune tolerance in the host. To address the foreseeable dilemma of unmet tissue demand, xenotransplantation, in vitro stem cell differentiation, and regeneration therapy of β-cells have been explored.

A. Prevention of Immune Destruction of Transplanted Islets

Islet graft rejection process can be divided into three categories depending on the etiology, severity, and the timing involved—hyperacute rejection, acute rejection, and chronic rejection (Rossini et al., 1999). Hyperacute rejection is the immediate rejection process that proceeds within hours and depends on preformed and primed antibodies within the host against the graft. This process is observed predominantly with xenotransplantation wherein preformed antibodies exist against the α-(1,3)-galactosyl residues present on endothelial cells of lower animals. The humoral response leads to complement fixation and thrombosis within minutes to hours of engraftment and finally to transplant failure (Groth et al., 1994). The acute rejection process depends on self- and nonself-recognition and is predominant with allotransplantation. This rejection process is characterized by rapid infiltration of immune cells, followed by T-cell responses. Immunosuppressive drugs inhibit the acute rejection process. The chronic rejection process, however, proceeds even in the presence of immunosuppression and is characterized by fibrosis and distortion of the architecture of transplanted tissue, leading to graft failure (Orloff et al., 1995). Various mechanisms have been proposed to account for the chronic rejection process. These include the wound healing process, delayed type hypersensitivity reaction, antibody-mediated humoral immunity, and endothelial cell damage (Rossini et al., 1999).

Various approaches have been attempted to target these different rejection processes. Hyperacute rejection of xenografts can be obviated with use of animals knocked out for specific genes, e.g., α-(1,3)-galactose, or a component for complement fixation (White and Yannoutsos, 1996) or use of complement inhibitors, complement depletion, and plasmapheresis of the recipient to remove natural antibodies (Rossini et al., 1999). The acute rejection process has been addressed predominantly through the use of immunosuppressant drugs, whereas chronic rejection is being addressed through the use of tolerogenic strategies (Womer et al., 2001a). The combination of immunosuppression and tolerance approaches is now being proposed for improved clinical outcomes in both islet and solid organ transplantation (Adams et al., 2001).

1. Immunosuppression. Generalized immunosuppression of the transplant recipient is the standard protocol today to prevent graft rejection by the host immune system. The first generation drugs that were applied to this end, include azathioprine, glucocorticoids, and antilymphocyte serum (ALS). Azathioprine is a calcineurin inhibitor. Calcineurin is a cytosolic calcium-dependent serine/threonine phosphatase protein that acts to remove phosphates from cytoplasmic regulatory proteins, which then penetrate the nucleus and act as transcription factors. Inhibition of calcineurin activity leads to inhibition of production of various cytokines including IL-2 and other gene products essential for T-cell activation. Although they are highly effective, these drugs have significant toxicity. Nephrotoxicity is prevalent in three-fourths of all patients (Burke et al., 1994). Additional side effects include hypertension, hepatotoxicity, neurotoxicity, hirsutism, gingival hyperplasia, and gastrointestinal toxicity. Other agents widely used for immunosuppression include glucocorticoids. These act through inhibiting T-cell proliferation and expression of genes encoding specific cytokines. They further block IL-2 production and also act by nonspecific inflammatory and antiadhesion effects. Their long-term administration, however, is associated with severe toxic effects including ulcers, hyperglycemia, osteoporosis, and increased risk of infection and neoplasms (Corbett et al., 1993; Diasio and LoBuglio, 1996; Dantal et al., 1998; Newstead, 1998). Antilymphocyte serum, e.g., polyclonal antithymocyte globulins to deplete T-cells in the host, is also widely used for immunosuppression during organ transplantation (Beiras-Fernandez et al., 2003).

Although some second-generation drugs are still used, those with higher potency and larger therapeutic window have been added to the drug cocktail. These include cyclosporine and tacrolimus (FK506) (Rossini et al., 1999). Cyclosporine acts on T-lymphocytes by forming a heterodimeric complex with cytoplasmic receptor protein, cyclophilin. Tacrolimus, on the other hand, binds to a cytosolic protein called FK 506-binding protein (Diasio and LoBuglio, 1996). However, many of these agents, including tacrolimus, cyclosporine, and steroids, are diabetogenic and toxic to the islets (Drachenberg et al., 1999). The use of immunosuppressive agents that do not challenge the islet graft is thus warranted. Although newer agents are constantly being developed, e.g., FTY720 (Fu et al., 2002) and lisofylline (Yang et al.,

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2004), improvements have also been reported with novel combinations of existing agents.

The islet transplant center at the University of Alberta in Edmonton, AL, Canada, has reported a high success rate of islet allotransplantation by sequential islet transplantation 2 to 10 weeks apart using two or more pancreases to achieve adequate mass of engrafted islets and by using a glucocorticoid-free immunosuppressive regimen that includes IL-2 receptor antibody (daclizumab), sirolimus (rapamycin), and low-dose tacrolimus (Shapiro et al., 2000). Daclizumab is given intravenously right after transplantation and then is discontinued. Sirolimus and tacrolimus must be taken for life. Daclizumab does not adversely affect islet function or glucose metabolism (Bretzel, 2003). The Edmonton group reported insulin independence in all seven patients with transplantation of an islet mass of ~11,500 IE (basal diameter of 150 μm) per kg b.wt. In a follow-up study, this group reported an 80% success rate in terms of insulin independence for 1 year, which was maintained by 12 of 15 patients transplanted with 9000 IE/kg b.wt. (Ryan et al., 2001, 2002). Based on these encouraging results, a multicenter clinical trial to coordinate the implementation of the Edmonton protocol, called the Immune Tolerance Network, was initiated by the National Institutes of Health together with Juvenile Diabetes Foundation International with seven centers in the United States and Canada, and three in Europe (Bluestone and Matthews, 2000; Bluestone et al., 2000). The Immune Tolerance Network sponsors investigator-initiated research in targeted prevention of immune-mediated transplant rejection by blocking immune signals at three different levels: T-cell recognition of antigen/MHC complex on APCs, costimulation to augment T-cell proliferative response to antigenic stimuli, and targeting clonal activation/deletion. One of these trials reported an insulin independence rate of 90% at the end of 1 year and long-term graft function in all 31 of 31 patients receiving transplants (Ricordi et al., 2005).

2. Immune Modulation and Tolerance. An inspiration toward the possibility of avoiding immunosuppression of the recipient without accompanying graft loss came from early observations of selective graft acceptance of twin animals that share common placental circulation during gestation. Graft tolerance in this case was ascribed to the exposure of neonatal animals to foreign antigens (Billingham et al., 1953). Such a selective immunological acceptance of a "foreign" graft by the immune system is known as immune tolerance. Tolerance is defined as the specific immune nonresponsiveness to an immunogenic stimulus (Samstein and Platt, 2001). There are some underlying assumptions to this definition of tolerance. It presupposes that the recipient is immunocompetent and that the immune response to the desired transplant is only qualitatively different from an immune response to other foreign tissue or pathogens. These assumptions, however, may not hold true in many cases because of a more profound suppression of the immune system with the strategies used and the inability to actually assess third-party graft rejection. The clinical and experimental criterion mostly used for the success of an immune tolerance intervention is the prolonged survival of the graft without immunosuppression, whereas stress on histological evidence of the absence of chronic rejection is now increasing.

Immune tolerance to an allogeneic or xenogenic islet transplant can be achieved at various stages in immune system development. These approaches target the graft, the graft donor, or the host. Based on the mechanistic point of interference, they may be classified as modulation of transplant immunogenicity, removal of passenger leukocytes, or induction of transplant tolerance. These are summarized in Table 2 and discussed below.

a. Antibody pretreatment for xenografts. Islet graft rejection is a predominantly T-lymphocyte-mediated process that occurs by several postulated mechanisms, e.g., provision of an adherence signal to T-cells by binding to the graft, antibody-dependent cell-mediated cytotoxicity, formation of immune complexes that physically block the vasculature and impair graft function, and complement fixation (Rossini et al., 1999). Therefore, the use of anti-T-cell antibodies should minimize islet graft rejection. Antibodies against the graft, however, can also have a protective role by masking the donor MHC class

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TABLE 2
Immune tolerance interventions for islet transplantation

Target	Strategies
Passenger leukocytes	Low-temperature culture to deplete resident islet macrophages (Lacy et al., 1979)
	Administration of antibodies against MHC class II molecules (Faustman et al., 1981)
Interleukin-1	Generalized immunosuppression using steroids (Corbett et al., 1993)
	Ex vivo gene therapy using IL-1 receptor antagonist (Gysemans et al., 2003)
TNF-α	Administration of soluble TNF-α receptor (Farney et al., 1993)
	Adv transfection of inhibitor of TNF-α (TNFi) (Dobson et al., 2000)
Nitric oxide	Use of *N*-monomethyl arginine (Xenos et al., 1994)
	Ex vivo gene knockdown of inducible nitric-oxide synthase
T-cell activation	Anti-CD4 Ig to prevent T-cell activation (Shizuru et al., 1987; Lehmann et al., 1997)
	Anti-CD154 (anti-CD40L) Ig to cause T-cell anergy (Markees et al., 1998; Zheng et al., 1999)
	Intrathymic administration of MHC peptides (Chowdhury et al., 1998)
	Coimplantation of soluble Fas ligand to cause apoptosis of T-cells (Judge et al., 1998)
	Anti-CD45 antibody (Basadonna et al., 1998)
	Use of CTLA4-Ig fusion protein (Tran et al., 1997)

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I antigens. Coating with an antibody that does not elicit a host immune response has been used to protect islet grafts against immune destruction. This strategy was used by Faustman and Coe (1991) for human islet xenotransplantation by precoating the donor antigens in islet tissue with the variable region of an antibody against donor MHC I molecules. They observed graft survival and histological improvement beyond 200 days without the need for immunosuppression.

b. Removal of passenger leukocytes for allografts. In the direct antigen presentation pathway, donor APCs migrate to the host lymph nodes to present donor antigen. Therefore, this pathway can be blocked by removing donor APCs from the graft before transplantation. Faustman et al. (1981) applied this strategy to prevent islet allotransplant rejection by pretreating the graft with antisera and complement to remove donor passenger leukocytes. In another strategy, islets were cultured in vitro at a reduced temperature (24°C) for 7 days before transplantation, a process that apparently led to the removal of passenger leukocytes. This approach, in addition to a single injection of ALS, achieved islet allograft survival for >3 months without immunosuppression (Lacy et al., 1979; Chervonsky et al., 1997).

c. Cytokine modulation. Cytokines may play either a destructive or an immunomodulatory role in islet graft rejection. Cytokines that contribute to graft destruction directly or by activating effector cells include IL-1β, TNF-α, and IFN-γ; whereas the cytokines that may impair graft rejection include IL-4, IL-10, and TGF-β. Some cytokines are classified as Th1 or Th2-based on the T-helper ($CD4^+$) lymphocytes that produce them. Th1 cytokines include IL-2, IFN-γ, TNF-β, and IL-12; Th2 cytokines include IL-4, IL-5, and IL-10. Th1 cytokines activate both T-cells and macrophages and promote cellular immune responses that serve as terminal effector mechanisms. Th2 cytokines produce reactions that favor humoral, IgE-mediated allergic, and mucosal immune reactions (Antin and Ferrara, 1992; Mosmann and Sad, 1996; Nickerson et al., 1997).

Expression of immunoregulatory molecules from the islet grafts themselves by various gene transfer approaches during ex vivo islet culture is an attractive option for preventing islet graft rejection. For example, Gallichan et al. (1998) expressed IL-4 in islets using lentiviral vector for stable transfection. They observed absence of inflammatory infiltrates in grafts and, upon transplantation in diabetes prone mice, protection of animals from autoimmune insulitis and islet graft destruction. This was consistent with the observation of switching of islet-antigen-specific T-cell responses toward a Th2 phenotype. However, autoimmune disease recurrence was not prevented by IL-4 gene transfer to islets before transplantation into diabetic NOD mice using transiently expressing adenoviral (Adv) vectors despite a significant level of transgene expression (Smith et al., 1997).

Transfection of islets with Adv encoding interleukin-1 receptor antagonist prevented IL-1-mediated islet destruction and loss of islet function (Giannoukakis et al., 1999b). IL-1β antagonism is beneficial not only in the prevention of graft destruction, but also in the prevention of autoimmune insulitis and in the pathogenesis of diabetes. Other cytokines that have been evaluated toward this end include IL-10 (Benhamou et al., 1996) and TGF-β (Ise et al., 2004). TGF-β was reported to mediate the effects of anti-CD3 antibodies in NOD mice in abrogating autoimmunity (Belghith et al., 2003). TGF-β was also implicated in the beneficial effect of mitomycin C on islet xenograft survival in a rat-to-mouse model (Ise et al., 2004) and in the protective effect mediated by Sertoli cells in a mouse allotransplantation model (Suarez-Pinzon et al., 2000). In the latter study, islets were transplanted into the left renal capsule of diabetic NOD mice whereas Sertoli cells were transplanted under the right renal capsule. Improvement in the survival and function of islets in Sertoli cell-transplanted mice were correlated to elevated plasma levels of TGF-β and its production by Sertoli cells. Following this lead, Suarez-Pinzon et al. investigated whether Adv-mediated ex vivo transfection of islets with TGF-β improved the outcome of islet transplantation. NOD mouse islets were transfected with porcine latent TGF-β1 using Adv-TGF-β1 and Adv vector alone. TGF-β1 overexpression from the islets resulted in longer normoglycemia (median period of 22 days versus 7 days for control), reduced $CD45^+$ T-cell infiltration of the graft, and reduced apoptosis of transplanted β-cells (Suarez-Pinzon et al., 2002).

TGF-β is postulated to act by generating $CD4^+CD25^+$ regulatory T-cells (Tregs) from $CD4^+CD25^-$). Tregs are potent suppressors of innate inflammatory responses and have been shown to enhance syngeneic islet transplant survival. TGF-β2 was shown to induce Foxp3 expression in $CD4^+CD25^-$ T-cells resulting in $Foxp3^+$ cells that behave like conventional Tregs (Fu et al., 2004). A recent study has further shown that systemic TGFβ1 gene therapy by intravenous injection of Adv-TGFβ1 induces the production of $Foxp3^+$ cells that restores self-tolerance by inhibiting autoimmune-mediated destruction of islets in the pancreas of NOD mice (Luo et al., 2005). These authors also transplanted 500 syngeneic islets under the kidney capsule of these mice 7 to 14 days after Adv-TGFβ1 injection. Islet graft survival time was prolonged (50 days in Adv-TGFβ1 injected mice versus 17 days in Adv-control injected mice) and was associated with peri-islet mononuclear cell infiltrate staining positive for CD4, CD25, and Foxp3. These studies demonstrate a cytoprotective role of TGF-β that could be used for both reducing autoimmunity and inducing transplant tolerance.

Free radical (NO· and $O_2^{\cdot-}$)-induced β-cell death is initiated by macrophage secretion of cytokines IL-1β and TNF-α. The prominent role of TNF-α in stimulating the immune system indicates that antagonism of TNF-α

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receptor binding may protect islet grafts from cytokine-mediated destruction. TNF-α expression is up-regulated in inflamed islets during the development of type I diabetes (Held et al., 1990), soluble TNF receptor administration blocks TNF-α mediated dysfunction (Farney et al., 1993), and transgenic mice expressing soluble type 1 TNF receptors secreted from β-cells escape insulitis and diabetes (Hunger et al., 1997). Furthermore, TNF-α injection to NOD mice led to an earlier onset of disease, whereas administration of anti-TNF monoclonal antibody resulted in complete prevention of diabetes development (Yang et al., 1994a). These observations indicate that islet treatment to antagonize TNF-α might improve the outcome of islet transplantation. Thus, Dobson et al. (2000) investigated the utility Adv-mediated transfection of an inhibitor of TNF (TNFi), whereas Machen et al. (2004) explored the use of soluble type 1 TNF receptor-Ig fusion protein. Dobson et al. (2000) transfected human pancreatic islets with Adv-producing TNFi and transplanted 2000 IE under the kidney capsules of NOD-SCID mice. Fifteen days after transplantation, the mice were injected with human peripheral blood leukocytes (huPBL) or buffer control. TNFi-transfected islets exhibited improved graft survival and function. The authors observed that TNFi effectively limited damage to β-cells by huPBL, although leukocyte infiltration was not affected. In addition, no difference between TNFi-treated and -untreated groups was observed in mice not injected with huPBL (Dobson et al., 2000). TNFR-Ig transfection, on the other hand, was shown to reduce cytokine-induced apoptotic human islet death in vitro and prolongation of normoglycemia after allotransplantation of BALB/c mice islets in streptozotocin-induced diabetic C57BL/6 mice. Machen et al. (2004), however, did not analyze graft leukocyte infiltration (Machen et al., 2004). These studies strongly indicate the central role played by macrophage-secreted cytokines (IL-1β and TNF-α) in mediating islet graft dysfunction and death, as well as the potential utility of intercepting cytokine pathways in improving the success of islet transplantation.

d. Intrathymic islet or alloantigen injection. Allograft rejection is mediated primarily by T-lymphocytes, as against xenograft rejection wherein the humoral antibody component plays a dominant role. T-cells undergo maturation in the thymus gland before being released in blood, and thymus is where self- versus nonself-antigen recognition is implemented by both positive and negative selection. An attractive approach that stems from this mechanism is to inject alloantigens directly into the thymus before graft transplantation to develop selective graft tolerance (Chowdhury et al., 1998). Intrathymic injection of a limited number of islets, or spleen tissue for presenting alloantigens, induces host T-cell tolerance to the subsequent extrathymic islet allotransplantation. This strategy has been applied to the transplantation of islets (Posselt et al., 1990, 1993; Mayo et al., 1994), as well as other organs (Goss et al., 1992; Odorico et al., 1992; Ohzato and Monaco, 1992).

For example, Levy et al. (2002) have shown prolonged survival of intrathymically injected islets in dogs so that prolonged alloantigen exposure and induced donor-specific tolerance is not compromised by islet survival within the thymic environment. Thymic islet injection along with a single dose of ALS has also been shown to induce islet allotransplantation tolerance (Posselt et al., 1990). The follow-up reports by the same group used intrathymic inoculation at birth (Posselt et al., 1993) and cotransplantation with bone marrow cells (Mayo et al., 1994). In addition to direct islet injection for donor islet alloantigen recognition, Oluwole et al. (2001) demonstrated rat tolerance to islet allotransplantation by an indirect mechanism after intrathymic injection of myeloid or thymic dendritic cells that had been exposed to donor antigens. These strategies, however, may be limited to islet allotransplantation only. This is because, even in the absence of humoral response against α-(1,3)-galactose moiety, xenotransplantation involves a much stronger T-cell response that may not be effectively suppressed. Thus, Tran et al. (1999) did not achieve significant prolongation of rat to mouse islet xenograft survival by intrathymic xenoantigen injection, whereas mouse to mouse allograft survival was relatively prolonged.

e. Peripheral tolerance by T-cell inactivation or depletion. The phenomenon of peripheral tolerance to a foreign antigen invokes the presence of T-cells that have receptors for the foreign antigen but are somehow nonreactive to the antigen. The presence of this phenomenon was elegantly demonstrated by Ohashi et al. (1991) by producing transgenic mice with islets expressing the antigenic glycoproteins of lymphocytic choriomeningitis virus. The presence of this antigen on the islets did not elicit an immune destruction of the islets, implying tolerance. However, extraneous infection by the virus caused the activation of T-cells, leading to the destruction of both the islet graft and the virus-infected cells. In addition to its implications on the possible viral involvement in the pathogenesis of type I diabetes, this phenomenon clearly implied the possibility of having the host immune cells being peripherally nonreactive to the antigen, even when the antigen is present on the graft tissue and in the presence of reactive host immune cells (Ohashi et al., 1993).

Intervention of immunostimulation at various stages has been attempted using monoclonal antibodies against specific cell surface receptors. Monoclonal antibodies directed against cell surface molecules important in immune activation and response can be used to induce immune tolerance. CD3 molecules are present on the surface of all T-lymphocytes proximal to the antigen recognition complex of T-cell receptors. Blockade of CD3 by monoclonal antibodies can effectively prevent T-cell activation and response to antigen presentation. This

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line of therapy has been widely used in solid organ transplantation. In fact, OKT3 (muromonab), a murine anti-human CD3 monoclonal antibody, is a standard line of therapy for treating acute graft rejection in solid organ transplantation (Diasio and LoBuglio, 1996). It binds T-cells and prevents participation of T-cells in immune response, while also causing rapid depletion of total T-cells from blood.

Application of the anti-CD3 approach to achieve T-cell nonresponsiveness to immunostimulation during islet transplantation has been tested in rodents and monkeys [using immunotoxin (Contreras et al., 1999, 2000; Thomas et al., 2001)] and humans [using monoclonal antibody, (Hering et al., 2004)]. Anti-CD3 monoclonal antibodies have been used to aid the development of mixed chimerism in the NOD mouse model without using severe irradiation procedures (Liang et al., 2005). Contrearas et al. (1999) found that the side effects of anti-CD3 immunotoxin in rhesus monkeys were manageable. The benefit of immunotoxin administration to reduce the duration of conventional immunosuppression was evaluated in three nonhuman primate models of diabetes, using two as nontransplanted controls. The animals were immunosuppressed using anti-CD3-immunotoxin, cyclosporine A, and methylprednisolone for only 4 days. Complete glucose normalization was observed in all three islet transplant recipients up to 18 months post-transplantation (Contreras et al., 2000). A similar study in the streptozotocin-induced diabetic rhesus monkey model demonstrated that conventional immunosuppression was required for only 14 days (Thomas et al., 2001).

Alegre et al. (1995) developed a humanized anti-CD3 monoclonal antibody that lacks Fc-receptor binding activity through mutagenesis of amino acids in the Fc portion, resulting in a less antigenic protein therapeutic agent. In a human allotransplant setting, Hering et al. (2004) used Fc receptor nonbinding humanized anti-CD3 monoclonal antibody hOKT3γ1 (Ala-Ala) in combination with sirolimus and tacrolimus in diabetic patients. They achieved normoglycemia in four of six patients with prolonged $CD4^+$ T-cell lymphocytopenia, inverted CD4/CD8 ratios, and an increased percentage of $CD4^+CD25^+$ T-cells.

In addition, anti-CD4 antibodies may be used to target the $CD4^+$ T-cells, implicated in initiating islet xenograft as well as allograft rejection (Suarez-Pinzon et al., 1996; Yi et al., 2002). These antibodies could be both depleting (resulting in clonal deletion of naive $CD4^+$ T-cells) or nondepleting and could be acting through a myriad of mechanisms including immune deviation, suppressor cell activity induction, and anergy (Rossini et al., 1999). This concept was pioneered by Shizuru et al. (1987), who demonstrated prolonged islet allograft survival in diabetic mice coinjected with an antibody directed against the L3T4 surface antigen on $CD4^+$ T-cells. Other researchers have documented the presence of donor-reactive T-cells (Lehmann et al., 1997) and clonal anergy (Alters et al., 1991), while confirming the applicability of this strategy to islet transplantation.

f. Coactivation and costimulation blockade. T-lymphocytes require the engagement of the T-cell receptor, as well as a series of coreceptors, which provide accessory and/or costimulatory signals, for full activation. These processes are explained in detail in section II.C. and Fig. 3. Blockade of these coreceptors leads to incomplete activation and immune tolerance. Several strategies may be adopted to induce immune tolerance by this mechanism.

CD45 is a family of transmembrane tyrosine phosphatases intimately involved in T-cell receptor-mediated signal transduction (signal 1) (Basadonna et al., 1998). Use of monoclonal antibodies against CD45 was shown to prolong islet allograft survival in NOD-SCID mice injected with huPBL (Gregori et al., 2005). In addition, Auersvald et al. (1997) evaluated the use of anti-CD45 monoclonal antibodies for allograft survival in a mouse model of diabetes by islet transplantation under the kidney capsule. They observed prolonged islet allograft survival in anti-CD45 monoclonal antibody-treated animals on days −1, 0, and 5 of transplantation.

Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is a potent T-cell down-regulatory molecule that appears to be essential for the development of peripheral tolerance. CTLA-4 is fused with immunoglobulin G1 Fc region (CTLA-4-lg) to enhance its serum half-life. The CTLA-4-Ig fusion protein can promote allograft survival by interfering with B7-1 (CD80) and B7-2 (CD86):CD28 or CTLA-4-mediated costimulation by competitive binding with B7-1 and B7-2 molecules on the APCs (Fig. 3). Blocking costimulation in this manner prevents T-cell activation and mounting of the cellular antibody response. Lenschow et al. (1992) injected CTLA-4-lg to induce human to mouse xenograft tolerance. They hypothesized that CTLA-4-Ig therapy blocked human pancreatic islet rejection in mice by directly affecting T-cell recognition of $B7^+$ APCs. In an alternative approach, Chachine et al. (1995) demonstrated prolonged islet allograft survival in mice whose muscle cells were transfected to produce CTLA-4-Ig. Tran et al. (1997) used the injections of chimeric fusion protein of CTLA-4 with the heavy antibody chain (CTLA-4-Fc) to induce islet allograft tolerance in mice. The transplant tolerance could be-induced with CTLA-4-Fc alone and was resistant to a later injection of IL-2, a helper T-cell stimulant, indicating that anergy, rather than clonal deletion, was involved in CTLA-4-mediated immune tolerance.

A critical step of T-cell activation involves the binding of constitutively expressed CD40 on APCs with the transiently expressed CD154 or CD40 ligand on the T-cells. Using a monoclonal antibody against CD154 would block this coactivation mechanism (Fig. 3). This strategy has been combined with allogeneic small lymphocytes (Parker et al., 1995) or donor spleen cell administration

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(Gordon et al., 1998) to induce donor specific tolerance in the mouse allotransplantation model.

One of the promising methods to prevent islet graft rejection is to target cell adhesion molecules (Socha-Urbanek et al., 1998). Cell surface interactions are essential for lymphocyte trafficking, migration, and activation in inflammatory responses and transplant rejection (Barrou et al., 2002). Multiple receptor-ligand interactions mediate cell surface adhesion. The receptors include two adhesion molecule families: the integrin family and the Ig superfamily. Leukocyte function antigen-1 (LFA-1, CD11a) is a β_2 integrin with three known ligands: ICAM-1, -2, and -3 that are members of the Ig superfamily. Nicolls et al. (2000) have shown prolonged islet allograft survival in different strains of rats using a monoclonal antibody targeted against LFA-1 (Fig. 5). They transplanted 450 IE under the kidney capsule of streptozotocin-induced diabetic mice that were pretreated with anti-LFA-1 antibody at a dose of 100 μg/day over a 6-day period after transplantation, with rat IgG administration as a control (Nicolls et al., 2000). Normoglycemia was observed in mice with islet allotransplantation across three different strains in an anti-LFA-1 treated group, whereas the IgG group consistently failed to alleviate hyperglycemia.

Targeting the ligand of LFA-1, ICAM-1, using a monoclonal antibody may also be useful in preventing islet graft rejection. ICAM-1 is a cell surface glycoprotein expressed by endothelial cells of the islets upon transplantation. The role of ICAM-1 in transplanted tissue destruction stems from its nonspecific adhesion of T-cells, which promotes T-cell infiltration and interactions with the graft to cause rejection. In vitro pretreatment of human pancreatic islets with anti-ICAM-1 prolonged xenograft survival in mice. Lymphocyte infiltration was markedly reduced, and graft survival prolongation was not accompanied by systemic tolerance, indicating the local effect of ICAM-1 blockade (Zeng et al., 1994).

g. Dendritic cell infusion. Dendritic cells bind allogeneic T-cells and are potent APCs. Dendritic cell infusion has thus been used to induce allogeneic tolerance. Simultaneous injection of dendritic cells that express the immunoregulatory molecule CTLA-4-Ig prolonged islet allograft survival up to 20 days compared with 11 days in untreated control mice (O'Rourke et al., 2000). In another study, in which MHC class I peptide-pulsed host dendritic cells were injected 7 days before islet allotransplantation in mice, 100% islet allograft survival was achieved for >200 days compared with 15 days for control (Ali et al., 2000). In both cases, islet grafts derived from a different strain were rejected. These results demonstrate the potential of specific tolerance induction in facilitating islet transplantation without immunosuppression.

All of these strategies of inducing immune tolerance have been proven experimentally in rodents and small animals and to a more limited extent in large animals and mammals as well. Although interception of the immune system in one or more manners and at one or different mechanistic points of immune response is promising, their effectiveness as individual strategies is limited. Therefore, more than one immune modulation approach are commonly combined. For example, combining anti-CD45 antibody with CTLA-4 up-regulation was more effective in preventing islet allograft rejection (Ariyan et al., 2003). Another limitation with applying these strategies is the problem of endpoint detection. Graft survival and functionality are often the endpoints of all these treatment interventions, with immune cell infiltration being used to more directly assess T-cell response in some cases.

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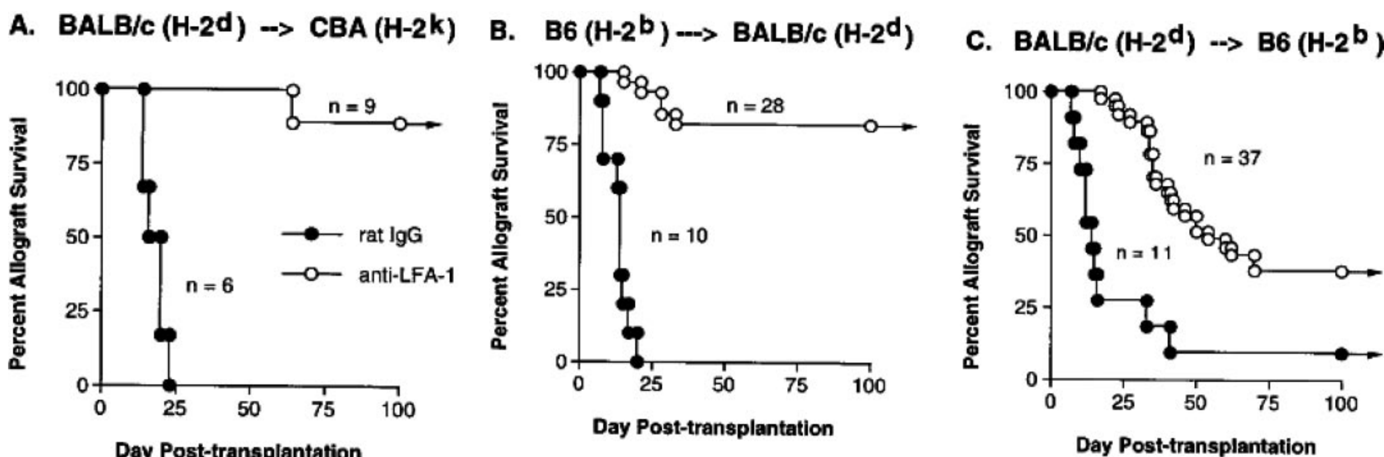

FIG. 5. Increased islet allograft survival with anti-LFA1 monoclonal antibody monotherapy in different allotransplantation models. Four hundred fifty islets were transplanted under the kidney capsule of streptozotocin-induced diabetic recipient mice of different strains: (A) BALB/c mice engrafted with islets from CBA mice, (B) B6 mice engrafted with islets from BALB/c mice, and (C) BALB/c mice engrafted with islets from B6 mice. Glycemic normalization is noted with anti-LFA1 antibody administration, whereas unrelated protein (IgG) administration does not lead to allograft survival. Reproduced from Nicolls et al. (2000) with permission. Copyright © 2000. The American Association of Immunologists, Inc.

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Another significant limitation of these approaches is their "incompatibility" with conventional immunosuppression (Adams et al., 2001). Whereas the former uses the immune system and relies on the functioning of its specific arms for effectiveness, the latter essentially suppresses the whole immune system. For ethical reasons, the first human trials of immune tolerance and immune intervention approaches would necessitate their coadministration with conventional approaches to safeguard the patient. Hence, it is of critical significance to identify those immune tolerance approaches that would be compatible and would act synergistically with existing immunosuppression strategies for organ and islet transplantation. The interested reader is referred to the review of Adams et al. (2001) for further discussion on this aspect.

B. Counteracting Insufficient Tissue Supply

1. Xenotransplantation. Transplantation of islets from nonhuman species has promise to overcome the potential problem of a limited supply of human islets, but the problem of immune reaction remains. Although various species have been evaluated as potential islet donors, the pig has been considered the primary alternative donor species. The pig has many favorable characteristics including its physiology and the similarity of porcine insulin to human insulin (difference of only one amino acid), and its having been widely used for human administration (Bretzel, 2003). One major barrier to pig islet transplantation in humans is the presence of the terminal carbohydrate epitope, Δ-1,3-galactosyl (gal) on the surface of pig cells (Cooper et al., 1993). This moiety is present in glycoconjugates of most mammals because of the enzyme α-(1,3)-galactosyl transferase, but humans lack this enzyme and have developed antibodies against α-gal from exposure to α-gal antigen on bacteria present in the gut such that >2% of total human IgM and IgG in the circulation represents α-gal antibody (McMorrow et al., 1997). This antigen has been implicated in the hyperacute rejection of pancreas xenotransplantation (Rayat et al., 1999). The generation of Δ-1,3-galactosyl transferase knockout pigs, however, has promise for overcoming this major problem of porcine islet rejection (Lai et al., 2002). Other sources of islets include cultured fetal porcine pancreas/islets and fetal pig proislets (Bretzel, 2003). These are often used instead of pig islets because of problems associated with pig islet isolation, as discussed in section II.A.

Another concern limiting xenotransplantation has been the risk of cross-species infection with porcine endogenous retroviruses (PERVs), which are permanently integrated in the pig genome (van der Laan et al., 2000). PERVs infect human cells in vitro (Patience et al., 1997), lead to permanent infection, and have been shown to cause cross-species infection. Immunosuppression of the host increases the risk of infection, whereas conflicting reports exist regarding potentiation by the depletion of α-gal antibodies (Rother et al., 1995; Moscoso et al., 2005).

Initial reports showed widespread PERV infection in immunocompromised mice originating from transplantation of infected pig islets (Ketchum et al., 2000; van der Laan et al., 2000; Clemenceau et al., 2002). A later study, however, indicated that PERV infection in peripheral tissues was associated with migration of porcine cells (Binette et al., 2004). In addition, exogenous forms of recombinant human-tropic PERVs have been identified in healthy swine, indicating that the risk of human infection may be more from the presence of these exogenous PERVs than from the replication of competent germline PERV loci (Wood et al., 2004). Furthermore, PERV infection was not detected in small laboratory animals (Specke et al., 2002a,b), nonhuman primates (Winkler et al., 2000; Switzer et al., 2001; Womer et al., 2001b), or humans exposed to live porcine tissues (Heneine et al., 1998, 2001; Paradis et al., 1999; Pitkin and Mullon, 1999; Dinsmore et al., 2000; Elliott et al., 2000; Abe et al., 2002). In a comprehensive in vivo study using a human-porcine chimeric mouse model, in which human and porcine tissues were transplanted simultaneously into immunocompromised mice, Yang et al. (2004) observed no PERV transmission to human cells, although the cells did get infected with a murine retrovirus. These studies indicate that the potential risk for human PERV infection after pig islet transplantation lies not in the porcine germline but in exogenous PERVs, which may be eliminated by controlled breeding of animals.

2. Regeneration Therapy. An important alternative to transplantation of pancreatic islets from human and xenogenic sources is the generation of insulin-producing β-cells either from pre-existing β-cells, or from non-β-cell precursors (Yamaoka, 2002, 2003; Ruggles et al., 2004; Trucco, 2005). In the former approach, a patient's own β-cells can be extracted and made to divide in culture before retransplantation into the patient. Various non-β-cells may also be used to generate β-cells: 1) the patient's own (adult) stem cells can be made to differentiate, 2) the patient's own terminally differentiated cells, e.g., pancreatic ductal cells, can be made to dedifferentiate into stem cells, followed by transformation into the β-cell phenotype, or 3) embryonic stem cells can be differentiated into insulin-producing cells. The use of a patient's own cells has the distinct advantage of circumventing immune problems, which is the most challenging barrier to successful islet transplantation. Although many details of our understanding of the differentiation pathway, as well as of the intricate mechanisms of islet function, remain unknown, significant progress has been made by several groups in generating cells of the desirable phenotype (Yamaoka, 2002, 2003; Ruggles et al., 2004; Trucco, 2005).

Three kinds of regeneration therapy have been identified for treating diabetes: in vitro, ex vivo, and in vivo

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(Yamaoka, 2002). In vitro regeneration therapy involves the generation of differentiated β-cells from cultured cells, such as embryonic stem (ES) cells, pancreatic stem cells, and the pancreatic duct epithelial cells containing islet progenitor cells. These cells are grown and made to differentiate in vitro, and the differentiated cells/tissue are implanted into the patient. A major shortcoming of this line of therapy is the need for lifelong immunosuppression. Ex vivo regeneration therapy, on the other hand, involves the removal of a patient's own cells, which undergo various treatments and are reimplanted into the patient. For example, a patient's own ES cells may be generated by nuclear transfer of the somatic cells into an anucleate oocyte from another person. In vivo regeneration therapy involves regenerating impaired tissues and cells from a patient's own cells in vivo. This may be achieved through two distinct approaches: inducing β-cell differentiation and stimulating β-cell growth. In another approach, promotion of physiologic regeneration by immune tolerance induction during diabetes development can be used to prevent the development of full-blown type I diabetes, as discussed in section III.B.2.a. Tissues from various organs of common origin (liver, pancreas, and intestine) contain pluripotent stem cells that may be induced to differentiate into insulin-secreting cell types (Yamaoka, 2002, 2003; Ruggles et al., 2004; Trucco, 2005). The rationale and applicability of these strategies are briefly discussed below and illustrated in Fig. 6.

a. Replication of pre-existing β-cells. An increase in β-cell mass has been recognized under a host of physiological and induced circumstances. Physiologically, islet mass of adult pancreas changes with pregnancy and obesity. Certain stimuli have also been shown to increase the islet mass: for example, partial pancreatectomy (Li et al., 2001), duct ligation (Wang et al., 1995b), cellophane wrapping of the gland (Swenne, 1983), alloxan- or streptozotocin-induced chemical damage (Yamamoto et al., 2000; Tourrel et al., 2001), or hyperglycemia (Bonner-Weir et al., 1989; Lipsett and Finegood, 2002). These observations of changes in β-cell mass have led to many questions. Does the β-cell mass increase by increased replication of pre-existing β-cells (regeneration or generation of β-cells from preexisting β-cells), decreased β-cell death, or differentiation of existing β-cell progenitors within or outside the islets (neogenesis or generation of new β-cells) (Trucco, 2005)? More importantly, can we exploit the same mechanisms to promote proliferation of β-cells in vivo for type II diabetes with reduced β-cell mass or in vitro for generating more β-cells for transplantation into type I diabetic recipients?

Various mechanisms have been proposed to be responsible for the increase in β-cell mass. These include the

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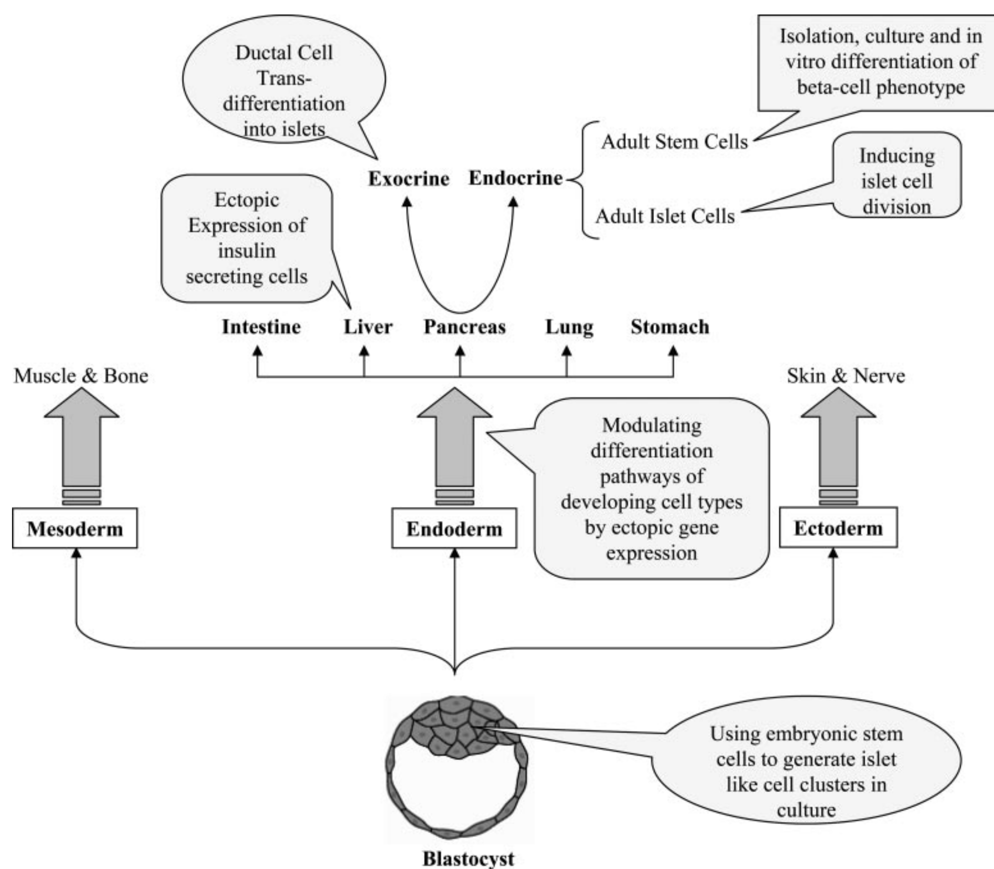

FIG. 6. Strategies for islet regeneration at various stages of embryological development of islets in the adult organ.

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differentiation of adult stem cells in exocrine pancreatic ductal cells, transdifferentiation of exocrine acinar cells of the pancreas, and increased replication or decreased apoptosis of preexisting β-cells of the islets themselves. Exocrine pancreatic ducts have been postulated to contain adult stem cells within the ductal epithelium, which may differentiate into the β-cell phenotype. β-Cells may also originate by transdifferentiation of acinar cells of exocrine pancreas via dedifferentiation to adult multipotent stem cells, which then give rise to the insulin-producing cells. Lipsett and Finegood (2002), for example, observed the generation of acinar-associated single β-cells, but no change in the duct-associated β-cell mass, when Sprague-Dawley rats were infused with 50% glucose solution for 3 to 4 days to maintain persistent hyperglycemia, compared with control rats infused with saline. This observation indicated that transdifferentiation of acinar cells into β-cells via dedifferentiation into neogenic focal areas is a possible mechanism for new β-cell formation in hyperglycemic rats.

The phenomenon of changes in islet mass under different physiological situations and by external stimuli suggests that the endocrine pancreatic cells, like other organ systems (e.g., blood, intestine), are in a continuous state of turnover and undergo dynamic changes with growth, development, and obesity. This turnover process would include neogenesis from adult pancreatic stem cells or regeneration from existing β-cells to increase the cell number, and apoptosis to reduce the number of cells. Exposure of islets to elevated glucose concentrations, for example, has a dual outcome on the turnover process. Glucose may induce or impair β-cell proliferation and may have pro- or antiapoptotic effects, depending on the duration of exposure and the genetic background of islets (Hoorens et al., 1996; Donath et al., 1999; Federici et al., 2001; Maedler et al., 2001). A switch in the outcome of glucose signaling from apoptosis to replication using a caspase-8 inhibitor of the apoptotic pathway (Maedler et al., 2002a) further demonstrated that the adult pancreatic β-cell mass is dynamic rather than fixed. This model is in line with the observation of limited (1–3 months) survival time of adult β-cells (Finegood et al., 1995).

Evidence for the replicative ability of β-cells within islets was seen in pancreatic development of insulin knockout transgenic mice (Duvillie et al., 2002). Transgenic mice were generated in which the two nonallelic insulin genes were disrupted ($Ins1^{-/-}$, $Ins2^{-/-}$) and lacZ was inserted at the *Ins2* locus to identify β-cells. A significant increase in the mean area of the islets was seen in embryonic and newborn mice compared with wild type and ($Ins1^{-/-}$, $Ins^{2+/-}$) controls, but the individual β-cells did not increase in size, suggesting an increase in β-cell mass due to an increase in cell number. The new β-cells could arise either from pre-existing β-cells after cell division (regeneration) or from pancreatic stem cells (neogenesis). The authors observed no change in the staining for proliferating cell nuclear antigen within pancreatic ductal epithelium and no increase in the number of β-cells associated with pancreatic ducts. Furthermore, the relative size distribution of islets was significantly increased in the transgenic mice. These observations suggest that the increase in β-cell mass is associated with increased replication of pre-existing β-cells, which leads to an increase in the size of existing islets rather than neogenesis of β-cells from adult stem cells. Further evidence that islets indeed undergo replication comes from cyclin D2$^{-/-}$ knockout transgenic mice. These mice have reduced pancreatic endocrine cell mass and demonstrate the necessity of cyclin D2 for replication of neonatal endocrine cells during pancreatic development (Georgia and Bhushan, 2004).

Further, in a landmark study by Dor et al. (2004) using a novel method for genetic lineage tracing, self-replication was shown to be the only predominant mode of adult β-cell formation. They generated a RIP-CreER transgenic mouse model in which Cre-estrogen receptor (Cre-ER) fusion protein was expressed under control of the rat insulin promoter (RIP). The expressed protein underwent nuclear translocation only in the presence of tamoxifen (Fig. 7A). A RIP-CreER mouse was crossed with the reporter Z/AP mouse model that houses a human placental alkaline phosphatase (HPAP) reporter under control of the cytomegalovirus (CMV)/β-actin promoter and blocked by lacZ expressing a loxP site (Fig. 7B). The double transgenic mice thus produced RIP-CreER;Z/AP underwent Cre-lox recombination in the nucleus of insulin-producing cells after tamoxifen stimulation. Thus, HPAP was expressed upon tamoxifen stimulation only in insulin-expressing cells.

The percentage of islets and individual β-cells originating from HPAP$^+$ precursor cells was determined after pulse-chase experiments. A stem cell or transdifferentiation model of β-cell origin, which would indicate loss of HPAP$^+$ cells (Fig. 7C), was not observed, thereby indicating that only pre-existing β-cells gave rise to new β-cells. This theory was proven in both the normal mouse model and the partial pancreatectomy mouse model. Whereas these results strongly contradict a hypothetical stem cell or transdifferentiation origin of new β-cells, which were hypothesized in histological analyses by many researchers, they also propose a strong in vivo proliferative potential of terminally differentiated β-cells (Dor et al., 2004). At the same time, however, the work of Dor et al. does not refute the presence of stem cells, per se, or the transdifferentiation ability of exocrine pancreatic cells, which could play a role under certain circumstances (Ohmori et al., 2005).

These observations imply that the utilization of β-cell mitotic factors and other stimuli that promote β-cell replication can be of tremendous value in the overall treatment of diabetes mellitus. Administration of such factors to type II diabetic patients with reduced numbers of islets who are undergoing further reductions in num-

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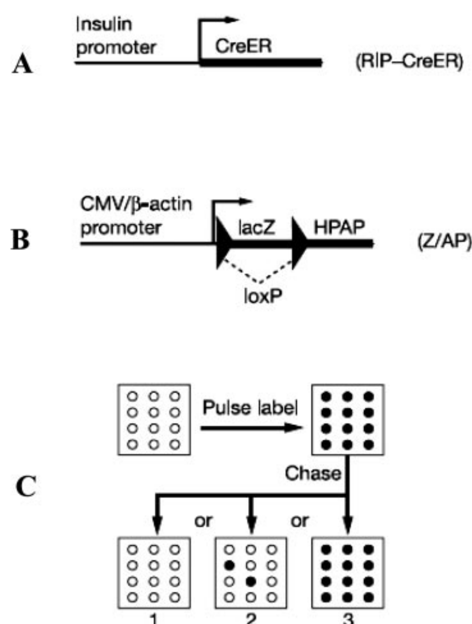

FIG. 7. Pulse-chase system for lineage tracing of adult pancreatic β-cells. Transgenic mice (called RIP-CreER) were generated in which (A) Cre-ER fusion protein was expressed under the control of RIP and was crossed with the (B) Z/AP transgenic mice in which HPAP reporter gene is expressed under the control of CMV/β-actin promoter. In the double transgenic mice produced, Cre-ER translocates into the nucleus after tamoxifen injection in insulin-producing cells, leading to Cre-lox recombination followed by HPAP expression. This system was used to trace the lineage of newly produced β-cells to assess (C) predictions from different models of β-cell maintenance. These models postulate that upon pulse labeling (HPAP$^+$) of all β-cells (represented by circles) in islets (represented by boxes), all β-cells will be HPAP$^+$ (indicated by ● to distinguish from HPAP$^-$ β-cells indicated by ○empty circles), assuming the labeling efficiency of 100%. After a chase period, entire stem cell-derived islets would contain no labeled cells (model 1), maintenance of β-cells within a given islet by adult stem cells would lead to a gradual decrease in the fraction of HPAP$^+$ cells (model 2), and maintenance by self-duplication would lead to the same fraction of HPAP$^+$ cells (model 3). These models were used with the pulse-chase labeling experiments for identifying the lineage of newly formed β-cells in adult mice pancreas. Reprinted by permission from Macmillan Publishers Ltd: Dor et al. (2004) *Nature* **429:** 41–46. Copyright © 2004 (http://www.nature.com/nature).

bers because of apoptosis may lead to restoration of islet mass and delay/prevent development of full-blown type I diabetes (Baggio and Drucker, 2002; List and Habener, 2004). Growth factors can also be used ex vivo. For example, hepatocyte growth factor (HGF) is a β-cell mitogen and has been reported to increase β-cell mass of isolated islets when used with extracellular matrix (Beattie et al., 1996, 1997). In the cell culture model, use of fibrin gels to preserve the three-dimensional structure of islets further improved HGF-mediated islet growth without loss of function (Beattie et al., 2002). To assess in vivo effects of HGF, Garcia-Ocana et al. (2000) generated transgenic mouse model expressing HGF under the control of rat insulin promoter. HGF overexpression was associated with 2- to 3-fold higher β-cell replication rates and 50% higher islet number per unit pancreatic area. They further observed higher insulin mRNA expression, increased insulin response to glucose and higher insulin content per cell, indicating that HGF causes β-cell proliferation and improves glucose response. Following this lead, they further tested the benefit of Adv-mediated ex vivo *HGF* gene transfer on the allotransplantation model using murine islets. Normalization of blood glucose was possible when a limited number of islets were transplanted, but normoglycemia was not achieved in control mice (Garcia-Ocana et al., 2003). The beneficial effect of HGF in terms of better blood glucose control persisted in the marginal-mass rat allogeneic islet transplant model without immunosuppression (Lopez-Talavera et al., 2004), indicating its potential utility in human clinical setting.

Ex vivo islet treatment with mitogenic factors can also enhance the outcome of islet transplantation, as also can cotransplantation of another cell type, prevention of cell death, or administration to the recipient after islet transplantation. For example, ex vivo transduction of islets with Adv expressing interleukin-1 receptor antagonist protein was shown to increase β-cell replication (Tellez et al., 2005). This increase was due to antagonism of the actions of IL-1β, which was shown to reduce β-cell replication to 10% of normal levels in adult rat islets. Although limited information is published on the effect of IL-1 on β-cell replication, this observation is significant in the "continuous turnover" model of islet cell homeostasis (Dor et al., 2004). According to this model, maintenance of islet cell count in adult life is a fine balance between islet cell death and regeneration. Thus, prevention of islet cell death by inhibiting the effects of IL-1 can improve β-cell count in islets.

The self-regeneration capacity of β-cells can also be used indirectly to prevent the development of full-blown diabetes. Autoimmune-mediated islet destruction and self-regenerative capacity of the pancreatic endocrine tissue counteract each other during the initial stages, until the autoimmune process supersedes and leads to the manifestation of diabetes in the patient. Abrogation of the autoimmune process at this critical stage can allow the regeneration process to take over, thereby preventing the development of overt diabetes. The applicability of this approach has been shown in the NOD mouse model, wherein prevention of autoimmune response by mixed allogeneic chimerism prevented autoimmune diabetes and reversed insulitis (Li et al., 1996; Zorina et al., 2003). The existence of this phenomenon of counteracting forces in humans is indicated by cases of spontaneous diabetes reversal (Karges et al., 2004). A detailed discussion of this approach is beyond the scope of this article. Interested readers are referred to an excellent recent review on this topic (Rood et al., 2006).

b. Ectopic expression of β-cell phenotype. Pancreatic and duodenal homeobox (PDX-1) is a major regulator of pancreas development and determines the function and phenotype of β-cells. It is expressed in all pancreas-dedicated cells of the endoderm during embryogenesis (Sander and German, 1997). Adenoviral delivery of *PDX-1* into mouse pancreas has been seen to induce ductal cell proliferation and β-cell neogenesis (Taniguchi

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et al., 2003). These studies have shown that it may be possible to generate insulin-producing cells in vitro without the need for isolating pure stem cells. Induced expression of *PDX-1* may be used for transdifferentiation of non-β-cells into the β-cell phenotype. This was achieved in hepatocytes using adenoviral gene transfer of *PDX-1*, which induced transdifferentiation of hepatocytes into insulin-secreting cells (Ferber et al., 2000). Insulin production by these cells ameliorated hyperglycemia in streptozotocin-induced diabetic mice. However, expression of *PDX-1* alone may not be sufficient to cause transdifferentiation of hepatocytes. Thus, when HepG2 cells were stably transfected in vitro followed by transplantation under the kidney capsule of streptozotocin-induced diabetic nude mice, the cells failed to transdifferentiate into insulin-producing cells and to normalize blood glucose levels (Lu et al., 2005).

On the other hand, in vivo growth stimulation of β-cells uses the physiological phenomenon of an increase in islet mass at certain times, e.g., pregnancy. Islet cell mass is known to increase in response to certain growth factors including insulin-like growth factor-I and -II (Robitaille et al., 2003; Lu et al., 2004), platelet-derived growth factor (Swenne et al., 1988; Welsh et al., 1990), growth hormone (Parsons et al., 1995; Liu et al., 2004), prolactin (Sorenson and Brelje, 1997; Amaral et al., 2003, 2004) and placental lactogen (Fujinaka et al., 2004). Hence, Adv-mediated expression of HGF has been used to improve the outcome of islet transplantation (Garcia-Ocana et al., 2003) and to reduce the number of islets required for successful glycemic control (Lopez-Talavera et al., 2004). Likewise, parathyroid hormone-related protein has been shown to have effects on either islet growth or insulin secretion capacity or both (Vasavada et al., 1996; Villanueva-Penacarrillo et al., 1999).

c. Using embryonic stem cells. ES cells are the inner cell mass of the blastocyst, which is formed during embryonic development of the neonate. To derive embryonic stem cells, in vitro fertilized eggs are allowed to develop into a blastocyst over 4 to 5 days. ES cells are isolated by transferring the inner cell mass onto a feeder layer of nondividing mouse embryonic skin cells. They are then cultured over several passages. Cells that have proliferated in cell culture for ≥6 months without differentiating are considered pluripotent ES cells. These cells have the unique ability to differentiate into any of the several different cell types of the adult animal, e.g., adipocytes (Dani et al., 1997), oocytes (Hubner et al., 2003), hepatocytes (Jones et al., 2002), neurons (Okabe et al., 1996), muscle cells (Rohwedel et al., 1994), cardiomyocytes (Wobus et al., 1991), and hematopoietic cells (Wiles and Keller, 1991; Nishikawa, 1997). Transplantation of cells differentiated from ES cells has been shown to restore the physiological function of many different organs including nerves (Brustle et al., 1999; Svendsen and Smith, 1999), spinal cord (Liu et al., 2000), and heart (Klug et al., 1996). Another attractive feature of the use of stem cells for regenerative therapy is the ability to manipulate them in culture including genetic modification and immunoisolation. Generating insulin-producing cells from embryonic stem cells has recently become a subject of intensive investigation.

Stem cells in suspension culture form multicellular structures called embryoid bodies. These bodies spontaneously differentiate into three embryonic germ layers— endoderm, ectoderm, and mesoderm—that are destined to produce different organs and organ systems of the whole animal (Martin et al., 1977). Cells of the outer layer of embryoid bodies (corresponding to the embryonic endodermal layer), as well as the inner mass of cells, have been observed to express insulin (Assady et al., 2001; Shiroi et al., 2002; Moritoh et al., 2003). Undifferentiated embryonic stem cells have also been shown to express insulin during in vitro culture (Soria et al., 2000). These observations indicate that isolation or differentiation of insulin-producing cells from embryonic stem cells or embryoid bodies, respectively, could be exploited for transplantation in diabetic recipients. Both these approaches have been tried.

Soria et al. (2000) stably transfected undifferentiated stem cells with a fusion gene consisting of phosphoglycerate kinase-hygromycin resistance gene and cDNA encoding human insulin/β-galactosidase gene. The resultant cell lines were differentiated in vitro, and insulin-secreting cell clones were isolated using a cell trapping system (Soria et al., 2000). The resulting clone displayed regulated glucose-stimulated insulin secretion in vitro and reversed hyperglycemia when 1×10^6 cells were transplanted in the spleen of streptozotocin-induced diabetic mice (Fig. 8). However, the normalization was reversible in 40% of ES-implanted mice that became hyperglycemic 12 weeks after implantation (Fig. 8B). Despite being hyperglycemic, these animals maintained their body weight and survived longer than sham-operated diabetic mice. Although the authors did not analyze the degree of differentiation of ES cells, theirs was the first study to demonstrate the potential of ES cell transplantation (Street et al., 2004b).

In vitro differentiation of ES cells into pancreatic islet-like structures was first reported in 2001, when Lumelsky et al. (2001) grew ES cells in culture and produced a highly enriched population of nestin-positive cells from embryoid bodies. Nestin is an intermediate filament protein that is normally found in neural precursor cells (Lendahl et al., 1990) and is one of the early markers of neural differentiation of ES cells. Both the adult pancreas and the central nervous system have similar developmental origin and involve the expression of nestin at one stage of embryonic development. Nestin-positive cells have been proposed as precursors to pancreatic islets (Hunziker and Stein, 2000; Zulewski et al., 2001). These nestin-positive cells were enriched by plating embryoid bodies in a serum-free medium in which many other cell types die, followed by expansion in the pres-

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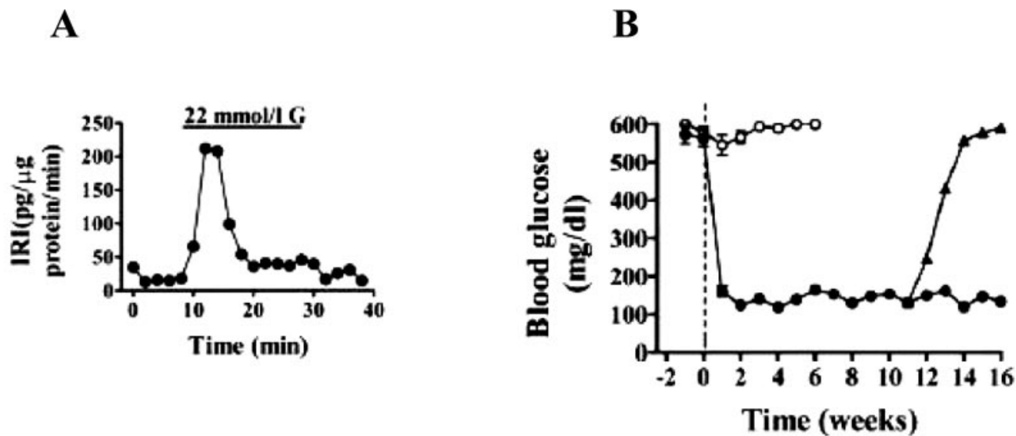

FIG. 8. Insulin secretion from embryonic stem cell-derived cells in response to changes in glucose concentrations. A, results of in vitro static incubation of 1×10^5 cells perifused at 1 ml/min with Krebs' buffer containing 1% bovine serum albumin and 3 mM glucose for 30 min for stabilization followed sequentially by 10, 30, and 10 min of perifusion with 3, 22.2, and 3 mM glucose containing Krebs' buffer in quadruplicate. Insulin concentrations in the incubation media were measured by radioimmunoassay. B, mean (±S.E.) blood glucose levels after transplantation of these cells (1×10^5 cells, $n = 4$) in the spleen of streptozotocin-induced diabetic mice ($n = 4$). Although the blood glucose levels in sham-operated mice (represented by ○; $n = 4$) did not normalize, ES cell-implanted mice became normoglycemic 1 week after transplantation (indicated by the arrow) in streptozotocin-induced diabetic mice. Some ES cell-implanted mice remained normoglycemic in the long run (represented by ●; $n = 15$), and others became hyperglycemic at week 12 (represented by ▲; $n = 6$). Reproduced from Soria et al. (2000). Copyright © 2000 American Diabetes Association. From *Diabetes*, vol. 49, 2000;157–162. Reprinted with permission from *The American Diabetes Association*.

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ence of mitogen, basic fibroblast growth factor, and mitogen withdrawal to cause cessation of cell division and differentiation. The capacity of these cells to store and release insulin increased with differentiation (Lumelsky et al., 2001).

Upon subcutaneous implantation in streptozotocin-induced diabetic mice, ES cells vascularized, formed aggregates similar to pancreatic islets, and remained insulin positive, although a sustained correction of hyperglycemia was not observed (Lumelsky et al., 2001). Researchers sought to overcome the low capacity of insulin storage and secretion by these cells by using growth inhibitors (Blyszczuk et al., 2003) and by stable expression of the *Pax4* gene (Blyszczuk et al., 2003), which is a transcription factor essential for β-cell differentiation during embryonic development (Soria, 2001). There have also been observations and arguments against using nestin-positive cells as β-cell progenitors (Lendahl et al., 1990; Selander and Edlund, 2002), as well as using insulin immunoreactivity as a marker of β-cell phenotype (Rajagopal et al., 2003). Furthermore, cell therapy of diabetes requires several considerations, such as control of cell number and cell differentiation in vivo and protection from host immune response. Immune protection of transplanted cells has been attempted by immunoisolation, induction of donor-specific tolerance, and genetic manipulations of donor cells to resist immune attack (Efrat, 1999).

An attractive option to in vitro differentiation of ES cells into islet or β-cell phenotype is the direct transplantation of undifferentiated ES cells into functionally compromised pancreas, wherein they will undergo differentiation into the endocrine phenotype by stimuli from the surrounding tissue (Street et al., 2004b). This approach, however, is fraught with the problems of potential tumorigenicity of transplanted cells. In addition, the use of ES cells is surrounded by many ethical and legal issues that have adversely affected research and clinical evaluation of these approaches. However, the recent demonstration of the capability of ES cells to differentiate into insulin-producing cells provides an alternative approach to the stem cell therapy of diabetes that, if pursued aggressively, is likely to overcome the problems associated with both immunity and tissue shortage.

d. Using adult stem cells. Islet neogenesis in adult pancreas under physiological conditions and stresses has been proposed to arise either from the preexisting β-cells or by the differentiation of adult stem cells. This hypothesis led to the search for adult pancreatic stem cells that may be successfully located and isolated from the diabetic patient for growing and differentiating in vitro to provide a desired amount of β-cell mass, or preferably preformed islets, to the patient. This would obviate the issues of alloimmune rejection, although autoimmunity may still remain a significant concern. Hence, this approach would have significant application for type II diabetes patients with reduced β-cell mass and for type I diabetes patients with adjunct interventions to sequester autoimmune antibodies. This approach would further bypass the ethical and legal concerns in using the human embryonic material required for ES cell-based therapeutics (Bonner-Weir et al., 2000; Ramiya et al., 2000; Zulewski et al., 2001).

Do pancreatic adult stem cells really exist? Where exactly are the adult stem cells located in the pancreas? How can we identify and isolate them? Answers to these questions have the potential of bringing this important

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therapeutic alternative to reality. In vitro expansion and differentiation of isolated cells can then be worked out using experience gained with ES cells. The search for answers to these questions, however, has not been very straightforward.

The existence of pancreatic stem cells is suggested by the high level of islet turnover in the pancreas coupled with the observations that terminally differentiated cells, including islets, do not normally undergo active proliferation (Street et al., 2004b). In the embryogenesis of the pancreas, the endodermal cells form a bud, which leads to sequential branching of ductal structures with cells that express the marker cytokeratin. Cytokeratin expression is eventually lost by these cells, and they form the exocrine and endocrine pancreas. Cytokeratin expression in cells of adult pancreas has been deemed to be indicative of the presence of pluripotent or multipotent stem cells. Direct evidence of islet neogenesis suggests that adult pancreatic stem cells may reside in the exocrine ductal epithelium or in close association with these cells. For example, individual β-cells, as well as intact islets, have been observed in close association with cytokeratin-positive ductal epithelium in the adult pancreas (Bouwens and Pipeleers, 1998; Bertelli et al., 2001). In addition, cells expressing both insulin and cytokeratin have been described in the adult pancreas (Bouwens and Pipeleers, 1998). Coexpression of these markers has been seen in vitro when ductal cells from adult pancreas were cultured as a monolayer (Bonner-Weir et al., 2000). Another marker that has been used to indicate the presence of stem cells in adult pancreatic ductal epithelium is *PDX-1* (introduced in section III.B.2.b.). Its expression, however, is gradually lost over the course of organogenesis, and in adult animals, only mature β-cells express *PDX-1* (Offield et al., 1996). Expression of *PDX-1* would thus be indicative of the presence of mature β-cells and/or the stem cells. *PDX-1* expression has been observed in premalignant ductal epithelium of transgenic mice overexpressing TGF-α (Song et al., 1999). Increased expression of *PDX-1* was also seen in rat ductal cells after partial pancreatectomy (Sharma et al., 1999) and in human pancreatic ducts both in vivo (Heimberg et al., 2000) and in culture (Rooman et al., 2000; Gmyr et al., 2001).

Can we generate insulin-producing cells in vitro from the ductal cells of mature pancreas? Delivery of genes identified in the embryogenesis of pancreas to ductal epithelial cells in culture has the potential to cause in vitro neogenesis of insulin-producing cells. For example, in vitro differentiation of the β-cell phenotype from ductal cells was shown by transduction with the early islet developmental transcription factor neurogenin 3 (Heremans et al., 2002). However, identifying and isolating stem cells remains a desirable objective. Toward this end, the expression of a marker cell surface protein may be used to isolate the desired cell type by, for example, antibody labeling with fluorescent probe and a flow-assisted cell-sorting technique. Exploration of such markers of adult pancreatic stem cells has led to the nerve growth factor Trk-A (Miralles et al., 1998), nerve growth factor (Rosenbaum et al., 1998; Teitelman et al., 1998), and the hyaluronan receptor CD44 (Satoh et al., 1997).

Thus, insulin-producing β-cells can be generated from adult pancreatic non-β-cells, although insulin-producing cells have not yet been produced in large quantities and tested in vivo for their efficiency. This technique offers the unique advantage of using cells originating from the transplant recipient, and thus obviating both the ethical and legal issues surrounding the use of embryonic stem cells, as well as the immune-mediated destruction of allogeneic and xenogenic islets.

3. Insulin-Producing Cell Lines. The β-cell lines developed by oncogenic transformation of quiescent, differentiated β-cells allow their expansion in culture to overcome problems of limited tissue supply while also obviating immune rejection when host β-cells are used for transformation. However, transplantation of cells that proliferate uncontrollably is an important safety concern since it may potentially lead to tumorigenesis and uncontrolled hypoglycemia. To address these issues, regulation of cell replication has been attempted using conditional oncogene expression by the use of the bacterial tetracycline resistance operon system such that the cells undergo growth arrest and maintain their differentiated characteristics in the presence of tetracycline (after transplantation), whereas they can be made to divide in the absence of tetracycline (in culture) to produce the desired number of cells. This was achieved by generating double-transgenic mice that expressed a transcriptional-activator fusion protein [tet repressor (tetR), with the activating domain of herpes simplex virus protein 16 (VP 16)] and an oncoprotein [simian virus 40 large tumor antigen (TAg)]. A cell line derived from these mice, which depended on oncoprotein expression for cell division, exhibited the characteristics of cell growth in the absence of and growth arrest in the presence of tetracycline (Efrat et al., 1995). This cell line, termed βTC-tet, was functionally effective during growth arrest and at various passages of cell culture (Fleischer et al., 1998). The use of a cell line, however, may still not be as efficacious as use of intact islets because of the intricate intraislet relationships between the cells that govern overall glucose homeostasis in the body (Samols et al., 1986; Caton et al., 2002).

IV. Biomaterial-Based Strategies for Improving the Success of Islet Transplantation

Applications for biomaterials in improving islet engraftment by immunoisolation of the transplanted tissue through semipermeable membranes are increasing. Immunoisolation prevents immune destruction of islets

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and facilitates the use of xenogenic and cell-based therapeutic options.

A. Immunoisolation of Transplanted Islets

Immunoisolation of transplanted islet tissue is an attractive strategy that addresses the problem of host immune destruction of transplanted tissue by blocking access of the host immune system. If successful, this strategy show promise to facilitate the use of alternative sources of insulin-producing cells, e.g., in vitro-generated β-cells and xenogenic islets for transplantation. Immunoisolation uses the enclosure of islets in a semipermeable membrane, which allows the passage of small molecules (e.g., insulin and glucose) but resists the entry of larger cells and antibodies of the immune system. Such a physical barrier could effectively inhibit islet destruction through both humoral- and T-cell-mediated immunity.

Three different kinds of encapsulated systems can be used for the purpose of islet transplantation: 1) perifusion chambers directly connected to the blood circulation (intravascular macrocapsules), 2) diffusion chambers in the shape of a tube or disk that can be implanted i.p. or s.c. (extravascular macrocapsules), or 3) the encapsulation of one or a few islets in globular membranes (extravascular microcapsules) (De Groot et al., 2004). Of all these three systems, microencapsulation of the islets has been investigated the most. Furthermore, hybrid systems are being investigated that use a combination of both macrocapsule- and microcapsule-based approaches to address problems associated with use of either system alone.

1. Types of Devices for Immunoisolation.

a. Intravascular macrocapsules. Intravascular macrocapsules are based on the principle of "dialysis cartridges," in which islets are seeded in the space between hollow fibers that are perfused with blood. The islets may be in a packed form or dispersed in a spacer matrix that prevents mutual adhesion and improves diffusional nutrient transport of the islets. These hollow fibers are enclosed within a larger tube, and the device is implanted into the vessels of the host by vascular anastomoses. Biomaterial used for the construction of these microcapillaries is polyacrylonitrile and polyvinylchloride copolymer, a biocompatible matrix often used in spinal cord injury. These devices permit close contact between the bloodstream and the islets, leading to efficient diffusional transport of metabolites. Encapsulation of islets in this device has been shown to induce normoglycemia in various animal models of diabetes including rats, dogs, and monkeys (Sun et al., 1977; Maki et al., 1993). The use of this device, however, requires intense systemic anticoagulation because of the direct contact of foreign material with blood and the potential for blood clotting, leading to potentially fatal thrombus formation, which is indicative of low biocompatibility of the implant material. An increase in the diameter of the capillaries led to increased flow rate of blood and reduced the risk of thromboembolism, but not without accompanying risks plus the complications associated with vascular prosthetic surgery (De Vos et al., 2002). These considerations shifted the research focus toward extravascular macrocapsules for islet engraftment.

b. Extravascular macrocapsules. Extravascular macrocapsules are based on the same principles as intravascular ones but have the advantage that biocompatibility issues do not pose a serious risk to the patient. These devices have been designed in both flat sheet membranous and hollow fiber formats (Zekorn et al., 1995). They can be implanted into the peritoneal cavity, the subcutaneous tissue, or under the kidney capsule. Various biomaterials have been used to generate these devices including nitrocellulose acetate, 2-hydroxyethyl methacrylate (HEMA), acrylonitrile, polyacrylonitrile and polyvinylchloride copolymer, sodium methallylsulfonate, and alginate. Biocompatibility of these devices is seen in terms of fibrosis at the site of implantation and covering the device. Various approaches have been used to enhance the biocompatibility of these devices, including the use of hollow fiber geometry because it offers reduced surface area of contact with the host per islet. Use of a smooth outer surface and hydrogels further improves biocompatibility of these devices by the absence of interfacial tension, thus reducing protein adsorption and cell adhesion. Hydrogels also provide higher permeability for low molecular weight nutrients and metabolites. Hydrogel materials that have been used include alginate (Simpson et al., 2005); agarose (Xu et al., 2001;Wang et al., 2002; Balamurugan et al., 2003), polyurethane (George et al., 2002b), chitosan-PVP hydrogels (Risbud et al., 2000), cellulose (Risbud and Bhonde, 2001), cross-linked hydrophilic poly(*N*,*N*-dimethyl acrylamide) with hydrophobic di-, tri-, and octamethacrylate telechelic polyisobutylene (Isayeva et al., 2003), and a copolymer of acrylonitrile and sodium methallylsulfonate (De Vos et al., 2002). Other approaches to address the problem of biocompatibility of these devices include membrane coating with poly(ethylene oxide) to reduce surface protein adsorption and surface hydrophobization with corona discharge. Surface fibrosis and biocompatibility remain the most significant hurdles to the successful use of both macrocapsule and microcapsule devices.

c. Extravascular microcapsules. Microcapsules enclose one or a few islets and are implanted at extravascular sites for obvious reasons. Microencapsulation of islets offers several advantages over macroencapsulation: higher surface area per unit volume for better diffusive transfer of nutrients and metabolites, mechanical stability, ease in manufacturing, and easy implanting procedures. Although various biomaterials have been used to produce microcapsules, the alginate-PLL system has been most widely investigated. In the typical process for microencapsulation of islets, isolated viable

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islets are suspended in a matrix (e.g., alginate solution), followed by droplet formation into a solution of cross-linking agent (e.g., divalent cation Ba^{2+} or Ca^{2+}, for alginate) that leads to surface sealing and formation of a semipermeable membrane. PLL has been used to impart semipermeable characteristics to the surface membrane and to enhance the integrity of microcapsules. This may be followed by a second layer of alginate to cover the free PLL groups. Morphological characteristics of microcapsules, as well as the challenges to their effective use in islet transplantation, are illustrated in Fig. 9. Thickness of the microcapsule, as well as the PLL barrier, interferes with oxygen and nutrient delivery. The lack of biocompatibility of PLL stimulates an inflammatory reaction on the surface of the capsules. Therefore, Omer et al. (2005) prepared alginate microcapsules by cross-linking with barium chloride without adding the PLL coating. Longer (~28 weeks) normoglycemic time was observed with alginate-$BaCl_2$ microcapsules (Omer et al., 2005) compared with ~10 to 15 weeks with alginate-PLL microcapsules (De Vos et al., 2003). $BaCl_2$ cross-linked microcapsules perform better than $CaCl_2$ cross-linked microcapsules, because barium cross-linkage results in stronger alginate gels than those made with calcium (Zekorn et al., 1992). The success of alginate microcapsules for islet transplantation is determined predominantly by purity and endotoxin content, as demonstrated by Omer et al. (2005) (discussed in section IV.A.3.). Several other biomaterials may also be used for islet encapsulation, e.g., HEMA, methacrylic acid, and methyl methacrylate (Chia et al., 2000, 2002; Sanders et al., 2005).

Encapsulated islets have shown improved graft function and survival compared with unencapsulated islets. Sun et al. (1996) have reported that the transplantation of encapsulated porcine islets in spontaneously diabetic monkeys induced normoglycemia without immunosuppression for more than 800 days. Schneider et al. (2005) have shown survival of encapsulated human and rat xenografts in mice for 7 months. These authors formed encapsulated islets in high-viscosity alginate in the presence of 3% human serum albumin by using air jet droplet formation. The droplet containing the islet was dropped into iso-osmolar barium chloride solution, followed by washing of formed microcapsules and removing excess barium (using sodium sulfate solution). These microcapsules had a diameter of 700 to 800 μm and lacked the PLL seal coat. Eighteen hundred encapsulated islets were transplanted into the peritoneal cavity of immunocompetent mice, and the animals were monitored for blood glucose levels. Normoglycemia was achieved for >7 months and islet viability of extracted microcapsules was >85%. The explanted islets had a minor cellular reaction and produced a normal glucose-stimulated insulin secretion response (Schneider et al., 2005). These results demonstrated the prolonged survival of encapsulated islets. Another report documented normoglycemia in a human patient with intraportal transplantation of microencapsulated islets for a period of 9 months (Soon-Shiong et al., 1994).

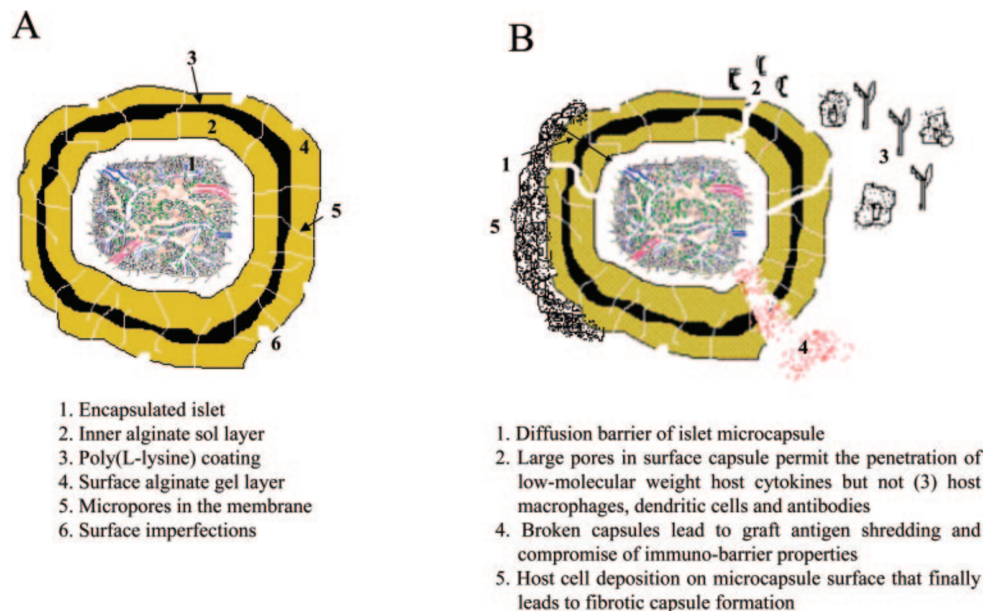

FIG. 9. Barrier properties of microcapsules and possible reasons for failure of encapsulated islet grafts. A, islet encapsulated in an alginate-poly(L-lysine) microcapsule with microporous membrane and surface imperfections in the microcapsule. B, some of the main reasons for the failure of islet grafts. These include (1) the large diffusive barrier that leads to limited nutrient and oxygen supply, (2) formation of large pores in the capsule through which low molecular weight host cytokines can penetrate, although (3) host antibodies, dendritic cells, and macrophages cannot, and (4) breakage of surface capsules that leads to donor antigen shredding and compromise of the immunobarrier with penetration of host cells and antibodies. Chemokine-mediated attraction of host mononuclear cells to the islet microcapsules, coupled with their inability to penetrate, leads to host-cell deposition on the microcapsule surface (5). This causes increased diffusional barrier thickness, a reduced nutrient supply to the islets because of nutrient consumption by these cells, increased exposure of encapsulated islets to the cytokines generated by these deposited cells, and, finally, the formation of a fibrotic capsule.

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2. Biocompatibility Considerations. The long-term survival and function of islets, however, is limited. Figure 9 shows survival barriers to immunoisolated islets by microencapsulation. Morphology of an encapsulated islet in an alginate-PLL microcapsule is illustrated in Fig. 9A, and Fig. 9B illustrates possible reasons for failure of islet grafts.

Primary impediments to the success of microencapsulation for islet transplantation include 1) biocompatibility, 2) inadequate immunoprotection, and 3) hypoxia. Inadequate biocompatibility is recognized by the pericapsular overgrowth on microcapsules that consists of fibroblasts and macrophages. Safley et al. (2005) observed inflammatory cell deposition around the microencapsulated porcine islet xenografts in NOD mice when the islet microcapsules were harvested from diabetic mice 15 days after transplantation (Fig. 10). Tissue overgrowth on the transplanted microcapsules directly correlated with islet graft failure (Safley et al., 2005), because such overgrowth is followed by fibrous tissue formation and leads to nutrient deprivation and inadequate diffusional transport from and to the islets, ultimately resulting in graft failure (De Groot et al., 2004).

There are three aspects of biocompatibility of transplanted microcapsules: 1) foreign body reaction of the recipient against the incorporated device, 2) reaction of the entrapped islet(s) to the encapsulation material and process residuals, and 3) immune reaction of the recipient against the encapsulated islet(s) (Zekorn et al., 1996). The foreign body reaction has often been termed "nonspecific" and arises because of the presence of impurities in the polymers used, e.g., monomers, catalysts, and initiators in synthetic polymers and mitogens and pyrogens in natural polymers (Chaikof, 1999).

The host reaction to the foreign material includes an immediate, acute, inflammatory response and a chronic, fibroblastic response. The former includes infiltration of immune cells with or without the eventual generation of a fibrous tissue in and around the implant site (Fig. 9). This pericapsular infiltrate, composed predominantly of macrophages and some helper T-cells, results in necrosis of the encapsulated islets. Importantly, immunosuppressive agents, e.g., cyclosporine, inhibit formation of this capsule (Wijsman et al., 1992). The fibrotic capsule consists of multinucleate fibroblasts, which generate a diffusive barrier to the flow of nutrients, oxygen, and metabolites. Formation of pericapsular overgrowth has been observed with the implantation of encapsulation material alone and has been regarded as a measure of biocompatibility (van Schilfgaarde and de Vos, 1999). In addition to the inherent properties of biomaterials themselves, pericapsular overgrowth has been attributed to imperfect encapsulation and chemotactic stimuli in the form of diffusible and permeable small molecules released from the islets. In the case of xenotransplantation, these diffusible stimuli also include antigenic substances such as α-(1,3)-galactose that attract and activate macrophages (De Vos et al., 1997; van Schilfgaarde and de Vos, 1999).

As illustrated in Fig. 9, inadequate immune protection of the islets results from the permeability of the barrier to small effector molecules of the immune system, e.g., NO, oxygen radicals, and inflammatory cytokines such as IL-1β, TNF-α, and IFN-γ from the macrophages and T-cells involved in the inflammatory reaction. Despite protection of islets from cellular engulfment, these small molecule effectors can destroy islets and lead to their nonfunction (De Vos et al., 1997; van Schilfgaarde and

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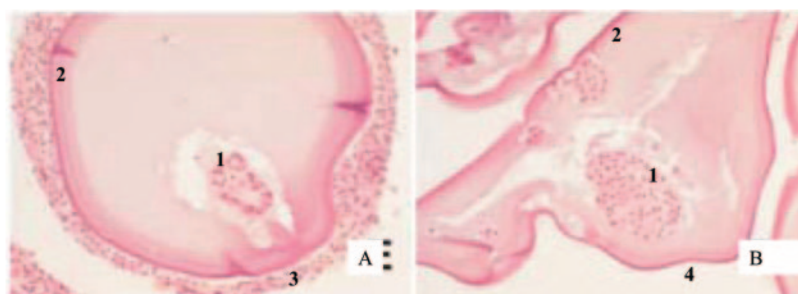

1. Encapsulated islet
2. Microcapsule boundary
3. Pericapsular overgrowth of fibrotic tissue and host immune cells
4. Islet capsule recovered from mouse treated with CTLA4-Ig and anti-CD154 monoclonal antibodies for immune tolerance induction did not have the pericapsular deposition

FIG. 10. Inflammatory cell deposition around the microencapsulated porcine islet xenografts in NOD mice correlates with the glycemic status of the animal and the use of immune tolerance induction strategies. The figure represents a hematoxylin- and eosin-stained section of islet microcapsules that were harvested from the mice and visualized by light microscopy at 400× resolution. A, rejected islet graft explanted at day 15 post-transplantation from a hyperglycemic mouse. B, functional islet graft recovered from a normoglycemic mouse at day 19 post-transplantation. The mice in group B received CTLA4-Ig and anti-CD154 monoclonal antibodies for immune tolerance induction by targeting CD28/B7 and CD40/CD40L interactions, respectively; the mice in group A did not. This figure was modified from "Inhibition of Cellular Immune Responses to Encapsulated Porcine Islet Xenografts by Simultaneous Blockade of Two Different Costimulatory Pathways" [Safley et al. (2005) *Transplantation* **79:**409–418].

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de Vos, 1999). Hypoxic cell death is another outcome of such capsule formation. Islets have an intricate vasculature that gets destroyed during the process of isolation and culture. This vasculature is rebuilt during the first few weeks of transplantation. However, the presence of a barrier membrane prevents the formation of blood vessels in and around islets, thus preventing revascularization of the tissue. Consequently, tissue nutrient and oxygen requirements are met, and metabolite removal processes occur, only through diffusive transport leading to hypoxic cell death of the core tissue. Hypoxia can have significant deleterious effects on cellular functions that require ATP-like insulin secretion, even though the cells may be alive at such reduced oxygen concentration (Dionne et al., 1993; Avgoustiniatos and Colton, 1997).

Parameters that affect the performance of transplanted tissue include the microenvironment at the site of transplantation, availability of nutrients and oxygen, and the extracellular matrix (Kemp et al., 1973). For example, the partial pressure of oxygen at an extravascular site (40 mm Hg) is considerably lower than that in the arterial circulation (100 mm Hg) (Chaikof, 1999). The development of fibrotic tissue over the implanted tissue exacerbates the diffusion problem by building a barrier and surrounding the encapsulated tissue with oxygen-consuming fibroblasts. Other factors that affect oxygen supply to the encapsulated tissue include oxygen consumption rate, geometry of the device, tissue density, and spacial arrangement of the encapsulated tissue (Avgoustiniatos and Colton, 1997).

3. Methods for Microencapsulation of Islets. Islet encapsulation requires special considerations because of the biological and highly delicate nature of this tissue. The encapsulation process, of necessity, has several constraints including aqueous dispersion with low agitation; the presence of isoosmotic salt, glucose, and oxygen in the media; controlled and physiologic pH of the encapsulation media; and a preference for a low encapsulation time. In the first report of islet encapsulation, the gelling property of natural, anionic polysaccharide alginic acid in the presence of calcium chloride was used (Lim and Sun, 1980). In a typical process, the formed microcapsules are postcoated with a cationic poly(amino acid), e.g., PLL or polyornithine, to improve capsule integrity (Chaikof, 1999). This is followed by a surface coating of alginate, resulting in a microcapsule morphology that includes encapsulated islet(s) in a sol layer of alginate, followed by PLL coating and the gel layer of alginate on the exterior (Fig. 9).

Three techniques have been used for islet microencapsulation and/or the fabrication of macrocapsule devices: 1) interfacial precipitation, 2) phase inversion, and 3) polyelectrolyte coacervation. The islet microencapsulation process uses the interfacial precipitation process predominantly, with gelling of a polyanionic polymer by a divalent cation, whereas most macrocapsule fabrication processes use polymer precipitation upon phase inversion. The polyelectrolyte coacervation process is a modification of the alginate-calcium chloride interfacial precipitation system, in which complexation of oppositely charged polymers leads to the formation of a hydrogel membrane (Chaikof, 1999).

Interfacial gelation between a polyanion and a multivalent cation involves suspending islets in an alginate solution, which is then mixed with a calcium chloride solution through either droplet generation by electrospray (Halle et al., 1994), submerged-jet, oscillating coextrusion nozzles (Sefton et al., 1997), or spinning-disk atomization (Senuma et al., 2000) or through a coaxial air flow system (Dawson et al., 1987) or conformal coating (Desmangles et al., 2001). These different processes have the same underlying principle and use cross-linking of anionic alginate by multivalent cations via electrostatic interactions. Alginate surface precipitation is followed by coating with the polycationic polymer such as PLL, which increases membrane integrity via electrostatic association with the anionic alginate. Various formulation parameters influence the physical properties of the membrane formed. For example, incubation time, concentration, and molecular weight of PLL have a significant impact on membrane resistance, thickness, and permeability (Gugerli et al., 2002). Membrane strength is further influenced by alginate composition and concentration (Thu et al., 1996). Interfacial gelation for encapsulation has also been achieved by free-radical cross-linking of functionalized polymers using photopolymerizable PEG-diacrylate (Cruise et al., 1998). This technique uses the cross-linking of derivatized PEG monomers through free radicals, which are generated by laser light activation of a photoinitiator (eosin Y) coated on the islet surface.

Although the alginate system for microencapsulation of islets has been used predominantly, it has significant limitations because of the possible contamination of pyrogens and mitogens and lot-to-lot variability. Alginate is composed of various sequences of α-L-gluconic acid (G) and β-D-mannuronic acid (M) arranged as homopolymers consisting of G and M residues alone or as copolymers of alternating G and M residues. Gel strength, rigidity, brittleness, and softness have been correlated to the proportion and block length of G homopolymers in the blend (Sun et al., 1996).

Omer et al. (2005) systematically investigated the role of G and M contents of alginate on biocompatibility, stability, and efficacy of transplanted encapsulated islets. Previous studies pointed toward the role of G (Clayton et al., 1991; De Vos et al., 1997) or M residues (Soon-Shiong et al., 1991, 1992) for the fibrotic reaction in transplanted encapsulated islets. These studies had used PLL coating with Ca^{2+} cross-linking on alginate microcapsules. Omer et al. (2005), on the other hand, had used barium ions to cross-link alginate in a single step. Duvivier-Kali et al. (2001) found high-M alginate

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moderately superior to high-G alginate in mice, although only a marginal difference was observed in rats (Omer et al., 2005). In larger animals, G and M contents did not significantly influence the biocompatibility or stability of microcapsules so long as high purity and low endotoxin content were maintained, as was reported previously (Zimmermann et al., 1992; Zhang et al., 2001). These observations could be due to species differences in biocompatibility and stability of microcapsules. Furthermore, encapsulated islet function in mice was better than that in rats, a finding that could be related to the number of islets transplanted per unit of animal weight, i.e., 30,000 IE/kg in mice versus 18,000 IE/kg in rats (Omer et al., 2005). Omer et al. (2005) further observed that smaller capsules (0.6 ± 0.1 mm versus 1.0 ± 0.2 mm) were better in terms of biocompatibility and stability. To assess in vivo function, the authors syngeneically transplanted 4000 IE/animal in a 1.7-ml buffer solution intraperitoneally into streptozotocin-induced diabetic Lewis rats. The animals were observed to be normoglycemic for >200 days.

Yoon et al. (1999) also tested the use of highly pure alginate-barium microcapsules in the xenotransplantation setting, which offers greater challenges in terms of host immune response. In this setting, they used porcine neonatal pancreatic cell clusters (NPCCs), which contain a high proportion of islet precursor cells that differentiate into β-cells after transplantation (Yoon et al., 1999). NPCCs, also called neonatal porcine islets, are preferred over adult porcine islets because of difficulties in isolating and culturing the latter (Omer et al., 2003). NPCCs restore normoglycemia after transplantation in streptozotocin-induced diabetic nude mice (Korsgren et al., 1990; Korbutt et al., 1996; Rayat et al., 2000). Omer et al. (2003) transplanted 10,000 IE of NPCCs in ~2.25 ml of buffer solution i.p. in streptozotocin-induced diabetic B6AF1 mice. In addition to monitoring the animals for weight, blood glucose levels, graft insulin content, and insulin secretory response, they also assessed cellular overgrowth, differentiation, maturation, and proliferation of β-cells in the NPCC microcapsules recovered from the animals 20 weeks post-transplantation. Euglycemia was achieved earlier (15 ± 12 days) (Omer et al., 2003) than reported previously (8 weeks) with alginate-calcium microcapsules (Rayat et al., 2000). Furthermore, normoglycemia was maintained for the duration of the study (20 weeks) without immunosuppression, and the encapsulated NPCCs differentiated and proliferated within the microcapsules. These results indicate that alginate capsules cross-linked with barium ions could be useful for xenotransplantation as well.

The polyelectrolyte coacervation process is based on a principle similar to the alginate-calcium chloride system but uses the complexation of oppositely charged polymers to form an interpenetrating network at the islet surface. Examples of such binary blends include alginate with protamine or spermine, carboxymethylcellulose with chitosan or dimethylaminoethyl dextran, and cellulose sulfate with poly(diallyldimethyl ammonium chloride). As expected, formulation and process variables influence the diffusional and other physical characteristics of such membranes. These include osmotic conditions, diluents, and molecular weight distributions of polymers used (Matthew et al., 1993; Li, 1996; Quek et al., 2004). Inability to independently control the permeability and mechanical strength of these systems has been a significant limitation (Chaikof, 1999). Hybrid systems using multicomponent electrolyte blends have been proposed for overcoming these limitations. Such systems involve polycation-induced gelling of an anionic polymer followed by a diffusional controlled reaction with a positively charged polymer of broad molecular weight distribution. An example of such a system includes the alginate-cellulose sulfate-poly(methylene-coguanidine) admixture (Wang et al., 1997b).

The phase inversion technique has been used to generate semipermeable membranes with various porosities. Both ultrafiltration (2–50 nm pore size) and microfiltration (0.1–1 μm pore size) membranes, which are suitable for xenotransplantation and allotransplantation of islets, respectively, have been generated by phase inversion as discussed in section II.C. (Chaikof, 1999). The phase inversion process involves the polymer precipitation from a homogeneous solution by change in either temperature or solvent composition. The latter is achieved by adding a nonsolvent that is miscible with the solvent used to prepare polymer solution, resulting in precipitation of the polymer. The process may be performed in either a solvent bath, called the wet process, or in a saturated atmosphere of the solvent, called the dry process. A soluble diluent is added to the polymer solution to aid in the formation of pores. Various formulation and process parameters of this process that influence the membrane characteristics include polymer precipitation time, polymer-diluent compatibility, and diluent concentration. This technology has also been used for microencapsulation using the polymer poly(HEMA-methyl methacrylate copolymer) (Crooks et al., 1990; Sefton et al., 1997).

In summary, the various technological options for encapsulating islets address the needs of membrane porosity, strength, elasticity, consistency, diffusional capacity, and thickness through idiosyncratic determinants of formulation and process parameters. These various physicochemical characteristics desired for any encapsulating material are in addition to the biocompatibility and biodegradability concerns. Achieving an optimal balance of all these characteristics has promise for the development of islet encapsulation as a viable alternative to generalized and lifelong immunosuppression of the transplant recipient.

4. Enhancing the Performance of Microencapsulated Islets. The need for combining more than one mechanism for enhancing the outcome of islet transplantation has been well recognized. Several researchers have evaluated the benefit of combining islet encapsulation with

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one or more additive strategies to improve the survival and functioning of transplanted islets. These have included, for example, coencapsulation of various agents, pretreatment or genetic modification of islets before encapsulation, and surface modification of islet microcapsules (Bae, 2004). These strategies addressed the two most significant bottlenecks to the application of microcapsules, viz., 1) islet graft destruction due to immune response and diffusive limitation of nutrient and oxygen flow, and 2) limiting volume of encapsulated islets required for transplantation.

Synergizing immune tolerance induction with microencapsulation can achieve long-term islet graft survival and reduction of T-cell response against microencapsulated islet grafts. This was illustrated recently in a study in which porcine xenograft islet transplantation in NOD mice by encapsulation in alginate-PLL microcapsules was combined with recipient pretreatment with CTLA-4-Ig and anti-CD154 monoclonal antibodies for immune tolerance induction by targeting CD28/B7 and CD40-CD40 ligand interactions, respectively (Markees et al., 1998; Zheng et al., 1999; Safley et al., 2005). This immune modulatory treatment significantly reduced the accumulation of host inflammatory cells (macrophages, eosinophils, and $CD4^+$ and $CD8^+$ T-cells) at the graft site, inhibited IL-5 and IFN-γ production, and promoted graft survival over a 26-day period compared with treatment groups that were sham-operated and those that did not receive immune modulatory treatment. As shown in Fig. 10, the accumulation of immune cells around the islet microcapsular graft clearly correlated with the glycemic status of the animals (Safley et al., 2005).

Other mechanisms leading to the destruction of transplanted islets are oxidative stress, NO-mediated cytotoxicity, and hypoxia experienced by the islets. Strategies that combine the use of agents to prevent or reverse such damages along with islet microencapsulation have been found to be beneficial to islet transplantation. The problem of hypoxia is particularly damaging to the inner cell mass of islets (as discussed in section II.B.), because oxygen transport takes place predominantly by diffusion. An increase in oxygen tension in the immediate vicinity of the islets can reduce or ameliorate the amount of hypoxia experienced by the islets. To this end, Chae et al. (2002) coencapsulated hemoglobin cross-linked with difunctional PEG with the islets in an alginate-PLL capsule. This strategy improved long-term insulin secretion and viability of the encapsulated islets (Chae et al., 2002). In their subsequent work, the authors found that coencapsulation of cross-linked hemoglobin also protected the islets from NO-mediated islet dysfunction (Chae et al., 2004).

Another approach to address the problem of hypoxic cell damage following transplantation is to promote the development of host vasculature toward the islets. This has been attempted by coencapsulation of VEGF protein with the islets. Thus, Sigrist et al. (2003a,b) encapsulated rat islets, after immobilization in a collagen matrix, in the presence of VEGF. The islets were then xenotransplanted into the peritoneal cavity of diabetic mice. The animals were monitored for islet function and development of microvasculature. Increased vasculature to the transplant site, improved insulin secretion, and better glycemic control was observed in the VEGF coencapsulated group than in the control group of islets (Sigrist et al., 2003a,b).

A significant challenge to the clinical application of microencapsulated islets is the overall high volume of implants that would correspond to the islet mass required for transplantation. Although glycemic normalization in human subjects with the transplantation of encapsulated islets has been reported (Soon-Shiong et al., 1993, 1994), the high volume required for transplantation limits the transplantation site to the peritoneal cavity. In an attempt to reduce the number of islets, some researchers have focused on increasing the efficiency of insulin release from the islets. A logical approach in this direction is the use of oral hypoglycemic agents that act on the islets to enhance insulin secretory activity in type II hyperglycemic patients, e.g., the sulfonylurea drugs. Thus, Hwang et al. (1998) synthesized poly(*N*-vinyl-2-pyrrolidone)-cosulfonylurea succinyl poly(ethylene oxide), which is a copolymer of glyburide and PVP, in which sulfonyl urea is attached to PVP via the poly(ethylene oxide) linker. Preparation of a polymeric conjugate of the active drug is necessary to increase its size into the colloidal range so that it does not diffuse out after transplantation. Other critical requirements to the success of this technology include solubility in the cell culture medium and the physiologic milieu, as well as bioactivity of the encapsulated conjugate. Coencapsulation of this conjugate increased glucose-stimulated insulin secretion from the islets (Hwang et al., 1998), as well as from an insulinoma cell line, MIN6 (Park et al., 2001). However, the increase was seen only at basal and not at elevated glucose concentrations (Kim and Bae, 2004).

To overcome this limitation, Kim and colleagues explored the use of glucagon like peptide-1 (GLP-1) for coencapsulation with islets in polymeric conjugate form (Kim and Bae, 2004; Kim et al., 2005). GLP-1 increases glucose-stimulated insulin secretion, which is dependent on glucose concentrations (Kieffer and Habener, 1999; Perfetti and Merkel, 2000). Islets were encapsulated in the presence of a copolymer of PVP-acrylic acid grafted with PEG attached to GLP-1 [poly(*N*-vinylpyrrolidone)-coacrylic acid-g-PEG-GLP-1 (VAPG)]. Whereas viability of encapsulated islets was similar in all groups, islets encapsulated in the presence of VAPG showed better insulinotropic activity than GLP-1-zinc crystals coencapsulated and control islets. The stimulation of insulin secretion was also dependent on glucose concentration (Kim and Bae, 2004). In addition, VAPG increased the cyclic AMP level in rat islets in a glucose concentration-dependent manner, but it had lower affin-

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ity binding with islet surface receptors than unconjugated GLP-1 (Kim et al., 2005).

B. Surface Modification of Islets

Camouflaging the surface of islets instead of incorporating them in a membrane barrier (Fig. 11) is another approach to both immunoisolation and immunoprotection. This process involves attachment of polymeric, hydrophilic chains to the islet surface to achieve molecular coating of the islets. Surface modification of islets by bioconjugation can overcome several potential problems with islet encapsulation. The diffusional barrier of less resistance and reduced thickness can be generated around the islets, compared with an encapsulation membrane. A diffusional barrier >200 μm in length is deleterious to diffusive transport of nutrients and metabolites. Furthermore, fine surface coating of islets leads to reduced volume of tissue per IE, which makes transplanting islets into human subjects feasible via the portal venous route of administration (Lee et al., 2002).

Strategies for surface coating of islets essentially use linear hydrophilic polymers such as PEG with an activated functional group and a mild conjugation reaction (Fig. 11). PEG has been used for surface modification of several cells and devices for reducing plasma protein adsorption and platelet adhesion and making them nonimmunogenic, e.g., red blood cells (Murad et al., 1999b) and T-lymphocytes (Murad et al., 1999a). Surface coating of rat islets with PEG was first reported by Panza et al. (2000) and was subsequently shown to be cytoprotective for porcine islet xenotransplantation in diabetic SCID mice (Xie et al., 2005). Panza et al. (2000) used PEG 5000-monoisocyanate for conjugation, whereas Xie et al. (2005) used a more specific succinimidyl derivative of PEG. Although both of these couple to the amines on the surface of cell membranes, the isocyanate group is less specific in that it may react with water during coupling reaction. Panza et al. (2000) demonstrated that the viability of islets was not compromised upon PEGylation and that islets retained the in vitro insulin response to glucose stimulation activity. Xie et al. (2005) additionally demonstrated protection in vitro against human antibody/complement-induced cytotoxicity in coated porcine islets and in in vivo islet function in the diabetic SCID mice model.

Xie et al. (2005) further introduced the concept of albumin shielding of islets using a disuccinimidyl derivative that is attached on one end to the islets and on the other end to an albumin moiety (Xie et al., 2005). This concept harbors the possibility of modifying encapsulation technology to literally “build” capsules on the islet surface instead of “encapsulating” islets. Heterobifunctional PEGs can be conjugated to the islet surface followed by attachment of another moiety on the exposed end of the PEG chains, which can then be cross-linked to each other to result in a firm microcapsule. Formation of a microcapsule in this manner will obviate many problems associated with the processing technology of microencapsulation discussed earlier. A comparative evaluation of the existing microencapsulation technology

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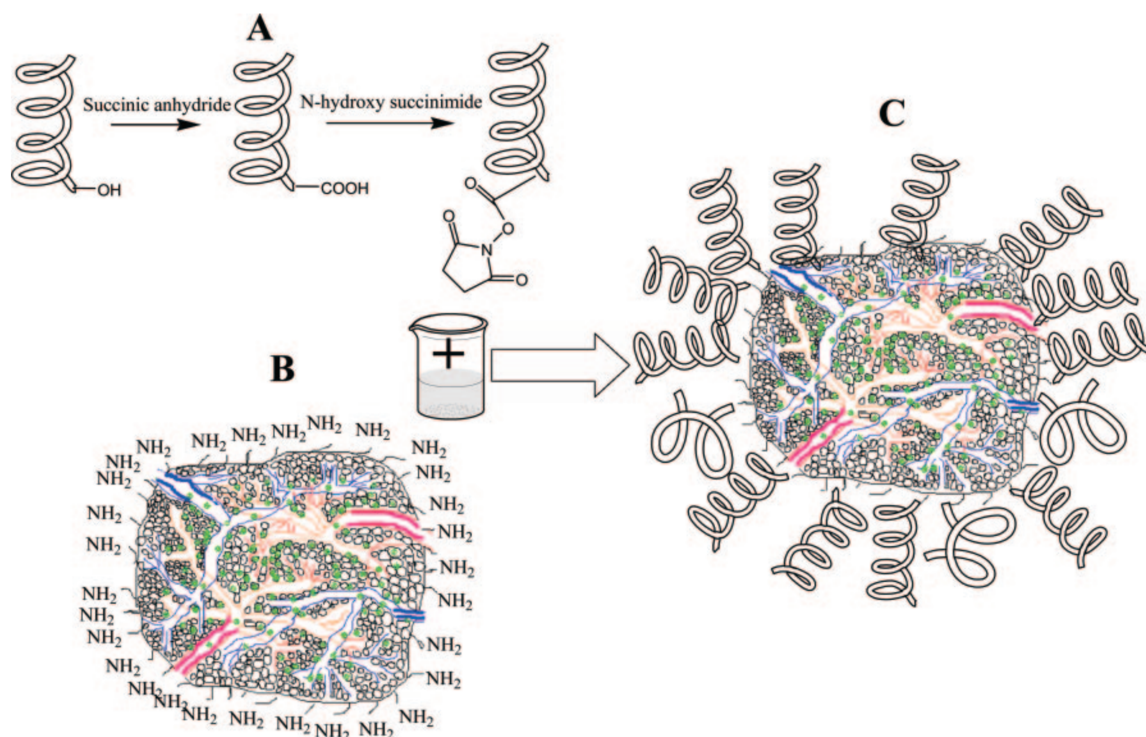

FIG. 11. Surface modification of islets with PEG. The terminal hydroxyl group of PEG is oxidized with succinic anhydride followed by activation with *N*-hydroxysuccinimide (A). The activated PEG is then conjugated to the cell surface amino groups (B) to result in a “coated layer” of PEG on the cell surface (C).

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with such an approach in terms of immunogenicity of encapsulated tissue is warranted.

In another study, Lee et al. (2002) PEGylated the surface extracellular matrix capsule of islets using methoxy poly(ethylene glycol)-succinimidyl propionate. They optimized reaction conditions for complete coverage of islet surface and maintenance of islet function following conjugation. Jang et al. (2004) demonstrated that coculture of PEG-coated islets with lymphocytes reduced the amount of secreted IL-2 and TNF-α. This finding indicated immune protection of islets, attributable to inhibition of release of diffusible antigens that activate lymphocytes. However, culturing of islets with macrophages did not lead to a significant difference from uncoated islets in terms of islet protection and the production of cytokines (IL-1β and TNF-α) and NO.

To address multipronged mechanisms of islet destruction, Contreras et al. (2004b) evaluated xenotransplantation of porcine islets in NOD-SCID mice after genetic modification of islets with the antiapoptotic gene *Bcl-2* and cell surface modification with PEG derivatives. This two-pronged approach promised to block host antibody binding to graft surface antigens due to hydrophilic PEG surface coating, while promising the cytoprotective effects of *Bcl-2* gene expression in the islets as demonstrated earlier (Contreras et al., 2001). The authors used PEG-mono-succinimidyl-succinate and di-succinimidyl-succinate after end-capping with albumin for surface coating of islets. Islet viability was maintained, and in vitro and in vivo islet functionality was not affected with these treatments (Fig. 12). The researchers further observed a reduction in complement-mediated cytotoxicity and an improvement in the blood glucose profile of transplanted mice, reflecting the benefit of combining the two approaches.

V. Nucleic Acid-Based Therapeutics for Improving the Success of Islet Transplantation

Therapeutic intervention is much needed to improve the outcomes of islet transplantation. These approaches target various mechanisms of islet graft rejection including immune- and inflammation-mediated destruction (Fig. 13), failure of revascularization and engraftment in the host tissue, and high islet mass requirement to achieve glycemic homeostasis of the host. The small-molecule drugs that target host immune systems are limited by their severe side effects, inadequate efficacy, and inability to selectively target the islet graft. Unlike small-molecule drugs and proteins, nucleic acid-based therapeutics offers a unique perspective of selectively expressing or inhibiting proteins identified as beneficial or deleterious to the islet graft, respectively. Furthermore, the process of ex vivo genetic modulation of islets in culture before transplantation offers a unique advantage of expressing desired proteins from the islets them-

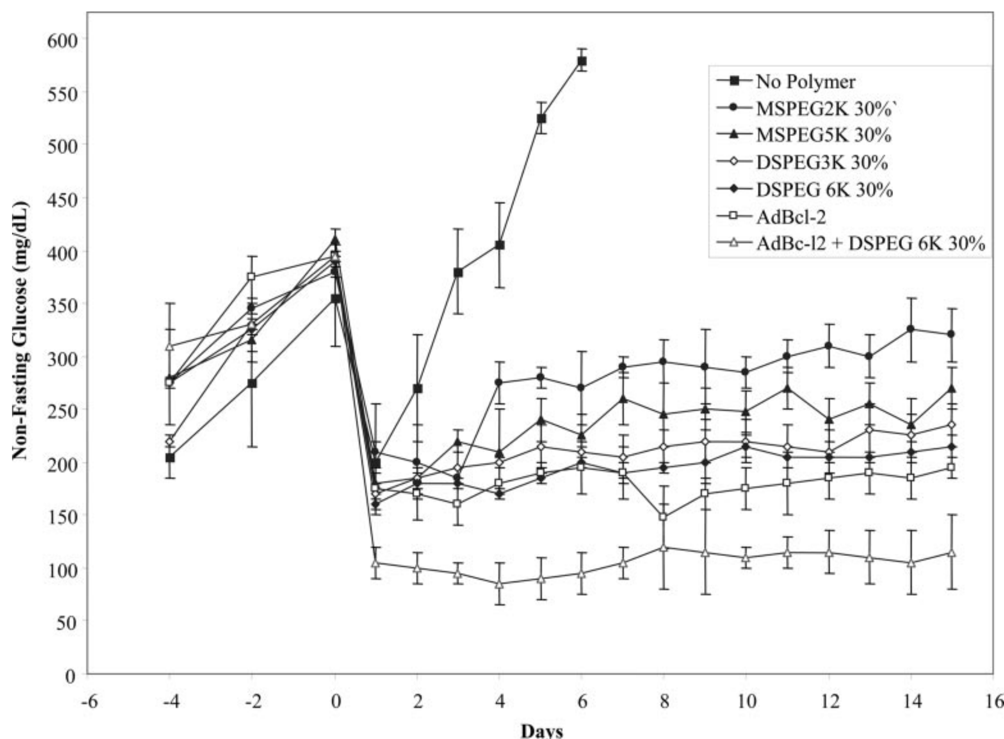

FIG. 12. Benefit of the dual strategy of antiapoptotic gene expression by adenoviral transfection and surface coating of islets with PEG. Improved glycemic normalization was noted in streptozotocin-induced diabetic NOD-SCID mice xenotransplanted with modified porcine islets in the hepatic portal vein. Increasing PEG size and combined use of *BclII* gene expression had a cumulative effect on islet function and blood glucose normalization in diabetic mice. Redrawn from *Surgery*, vol. 136, Contreras JL, Xie D, Mays J, Smyth CA, Eckstein C, Rahemtulla FG, Young CJ, Anthony Thompson J, Bilbao G, Curiel DT, and Eckhoff DE, “A Novel Approach to Xenotransplantation Combining Surface Engineering and Genetic Modification of Isolated Adult Porcine Islets,” 537–547, copyright © 2004, with permission from Elsevier.

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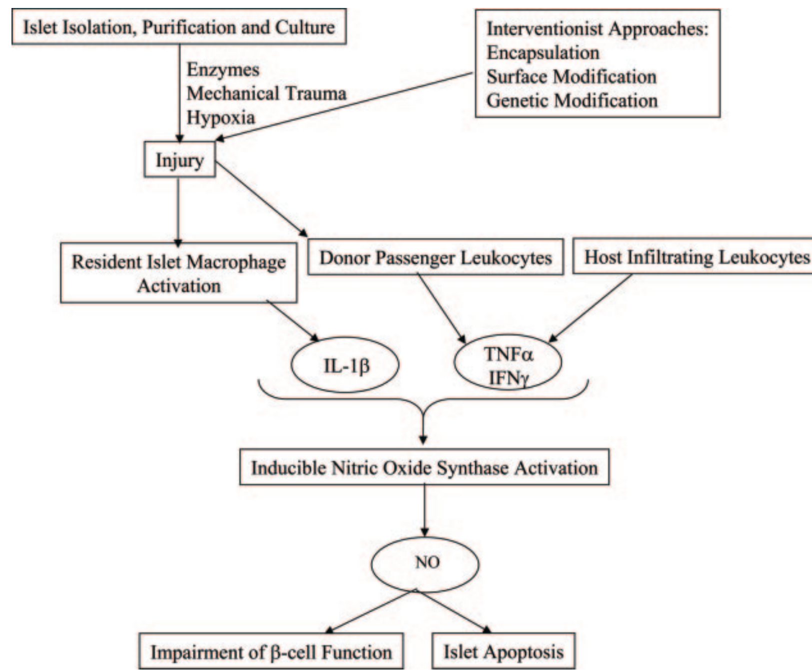

FIG. 13. Central role of iNOS in cytokine-mediated islet cell dysfunction and apoptosis. Injury due to islet isolation, purification, culture, transfection, and transplantation activates resident islet macrophages to release proinflammatory cytokines, such as TNF-α, IFN-γ and IL-1β, which leads to activation of iNOS gene and the release of cytotoxic NO.

selves, thus obviating problems associated with graft targeting, as well as systemic toxicity. Biomaterial-based approaches attempt to tackle these problems by islet surface modification and encapsulation but have additional constraints such as biocompatibility and increasing the diffusion barrier to nutrient and oxygen supply. These approaches, however, can be combined with islet genetic modification to address the limitations of each strategy and yield cumulative benefits.

Nucleic acid-based therapeutics can be used for either synthesis of proteins in the islets or gene silencing for inhibiting aberrant or undesirable protein production from the islets. These interventions have been used to enhance revascularization, to provide immune protection, and to prevent apoptosis of transplanted islets. In addition, in vivo gene transfer has also led to ectopic generation of insulin-producing cells, thus providing an alternative therapeutic possibility for the prevention and cure of type I diabetes. For example, expression of the insulin/pro-insulin gene from gut (Cheung et al., 2000), muscle (Riu et al., 2002; Shaw et al., 2002), and liver cells (Ferber et al., 2000; Lee et al., 2000) has been used to reverse diabetes. This review, however, will focus on the factors influencing gene transfer or silencing in the islets prior to transplantation.

Ex vivo gene transfer to isolated islets has been done using several gene candidates including VEGF (Mahato et al., 2003; Cheng et al., 2004; Narang et al., 2004) for revascularization and interleukin-1 receptor antagonist (Sandberg et al., 1993; Gysemans et al., 2003), TNF-α antagonist (Dobson et al., 2000), Bcl-2 (Rabinovitch et al., 1999; Contreras et al., 2001), A20 (Grey et al., 1999), heme oxygenase I (Pileggi et al., 2001; Tobiasch et al., 2001), IL-10 (Takayama et al., 1998), TGF-β (Lee et al., 1998), CTLA-4 Ig (Lu et al., 1999; O'Rourke et al., 2000), IL-4 (Chang and Prud'homme, 1999; Ko et al., 2001), IL-10 (Benhamou et al., 1996; Deng et al., 1997; Smith et al., 1997), IL-12 (Yasuda et al., 1998), IFN-γ receptor (Chang and Prud'homme, 1999), FasL (Kang et al., 1997; Judge et al., 1998), Adv E3 (von Herrath et al., 1997), insulin-like growth factor-I (Giannoukakis et al., 2000a; George et al., 2002a), dominant negative protein kinase Cδ (Carpenter et al., 2001, 2002), dominant negative MyD88 (Dupraz et al., 2000), nuclear factor κB (Wei and Zheng, 2003), inhibitor of κB repressor (Giannoukakis et al., 2000b), heat shock protein 70 (Burkart et al., 2000), manganese superoxide dismutase (Hohmeier et al., 1998), and catalase (Benhamou et al., 1998; Xu et al., 1999) for immune, inflammation, and apoptosis protection. The therapeutic utility and mechanism of intervention of these gene candidates has been discussed in section III. In the present section, we will focus on the delivery aspects of gene expression and gene silencing vectors in the context of ex vivo transfection of islets prior to transplantation.

Various nonviral and viral vectors have been used to transfect islets with desired therapeutic genes including Adv, adeno-associated virus, herpes simplex virus, and lentiviral vectors (Lachmann and Efstathiou, 1999; Vigna and Naldini, 2000; Stilwell and Samulski, 2003; Volpers and Kochanek, 2004; Somia, 2004). In the following sections, we will discuss various aspects of gene delivery to the islets.

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A. Nonviral Gene Delivery

The choice of nonviral and viral gene delivery systems for ex vivo transfection of islets prior to transplantation depends on the desired level and duration of gene expression. Depending on the gene candidate chosen and the therapeutic intervention intended, one may require prolonged or only transient gene expression. For example, up-regulation of expression of a pro-angiogenic gene like the VEGF (Mahato et al., 2003; Narang et al., 2004) will be required only for the initial few days of transplantation when islets undergo revascularization during the first 10 to 14 days after transplantation (Menger et al., 1994), whereas long-term expression and/or up-regulation of immune-modulatory genes such as IL-1 receptor antagonist (Giannoukakis et al., 1999a) may be useful in preventing islet graft rejection. Other considerations are the safety and toxicity to the islets. The latter include effects on islet function in terms of glucose-stimulated insulin secretion as well as islet viability as assessed by apoptotic and necrotic cell death upon such treatment (Mahato et al., 2003; Cheng et al., 2004; Narang et al., 2004). Transfection efficiency and toxicity of vectors are usually inversely related to each other. For example, although nonviral vectors are deemed safe, they have the disadvantage of low transfection efficiency. At the same time, Adv vectors have very high transfection efficiency, but pose the limitations of immunogenicity and transient expression (Giannoukakis et al., 1999a).

Bacterial plasmids have been the choice of vector for nonviral gene transfer. These are extrachromosomal elements in bacteria that are taken up by mammalian cells through endocytotic processes. This cellular uptake is enhanced when the plasmid DNA is complexed with mono- or polycationic lipids or polymers that act to condense the plasmid. The transfection efficiency, however, still remains much lower than in the recombinant virus-based systems. In addition, the transient nature of gene expression arising from metabolic instability of the plasmid in the cytosolic compartment and poor nuclear translocation are the predominant limitations of nonviral gene delivery. Several approaches have been tried to improve the outcome of plasmid-based gene delivery including modifications of the vector backbone and improvements in the gene carriers (Mahato et al., 1999; Mahato and Kim, 2002).

Vector backbone modification has focused on the role of various elements of gene expression systems. These include the promoter, the enhancer, the 3′- and 5′-untranslated regions, use of introns, and considerations in multiple gene expression, e.g., internal ribosomal entry site elements. The viral promoters and enhancers, especially those of CMV and simian virus 40, have been found to give maximal gene expression in most cell types including islets. Mammalian promoters that have found the most application include the β-actin and the ferritin promoters for these ubiquitously expressed genes. Cell targeting has been attempted using promoters, e.g., the insulin promoter (Odagiri et al., 1996; Welsh et al., 1999) and enhancers (Diedrich and Knepel, 1995; Sharma et al., 1997), e.g., the pancreatic islet cell-specific enhancer sequence (Beimesche et al., 1999), that are specifically expressed from β-cells. Plasmids containing two promoters or the internal ribosomal entry site element permit the simultaneous expression of two genes from the same plasmid, which is a desirable goal for the multipronged challenges of islet transplantation (Ko et al., 2001; Narang et al., 2004). The expression of both genes included in these bicistronic vectors, however, may not be to the same levels and would need to be evaluated for the vector elements chosen.

In terms of gene carriers, nonviral gene transfer to pancreatic islets has been done using both lipid, and polymer-based systems (Saldeen et al., 1996; Mahato et al., 2003; Narang et al., 2005). Other methods of nonviral gene transfer to islets, e.g., gene-gun technology (Gainer et al., 1997) and calcium phosphate precipitation (Saldeen et al., 1996), have proven to be relatively less effective and more damaging to the islets. Transfection efficiency of water-soluble polymer-based carriers is generally lower than that of cationic liposome-based lipid carriers. We have compared the transfection of Lipofectamine and Superfect in intact human pancreatic islets using an enhanced green fluorescent protein (EGFP) expressing plasmid, pCMS-EGFP. We found that the transfection efficiency obtained with the polymeric carrier was much lower than that of Lipofectamine (Mahato et al., 2003). In this study, we used the *VEGF* gene expressed under a chicken β-actin promoter for gene expression studies in human islets. We subsequently expressed the same gene under the viral CMV promoter in a modified bicistronic plasmid, pCMS-EGFP-VEGF, which had a higher level of gene expression (Fig. 14A) (Narang et al., 2004). *VEGF* gene expression from the plasmid vectors was evaluated at the optimal DNA dose (2.5 μg/1000 IE) and cationic lipid (Lipofectamine)/pDNA ratio (31±) over a period of 10 days using nontransfected islets as well as pDNA alone as controls. The expression of VEGF protein in the culture medium from 1000 IE transfected with the optimal plasmid dose and liposome formulation was 4 times higher for the gene expressed under the control of the CMV promoter in the bicistronic plasmid pCMS-EGFP-hVEGF (~6 ng/1000 IE) than for the β-actin promoter in the monocistronic plasmid pCAGGS-hVEGF (~1.5 ng/1000 IE) (Fig. 14A). The increased gene expression under the CMV promoter compared with β-actin promoter could be due to different activities of these promoters in the islet cells (Narang et al., 2004).

Human islets transfected with this modified vector were transplanted under the kidney capsule of NOD-SCID mice. Neovasculature development at the islet transplant site was assessed by immunohistochemical

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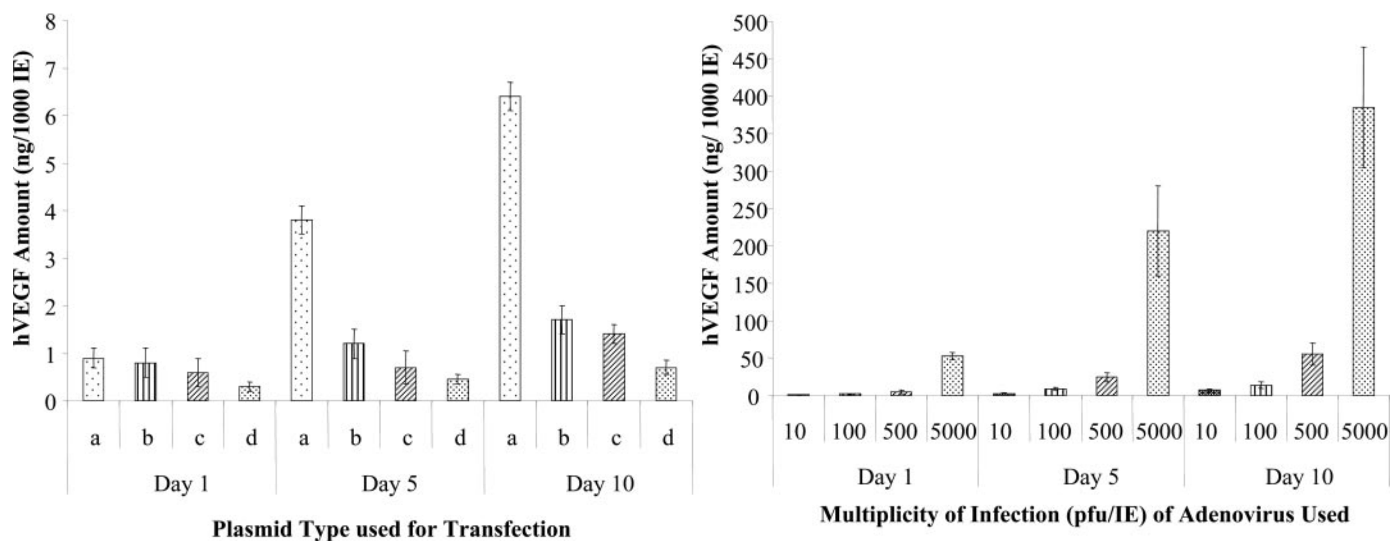

FIG. 14. Transgene expression after transfection of human islets with nonviral and adenoviral vectors. hVEGF encoding plasmid vectors pCMS-EGFP-hVEGF (a) and pCAGGS-hVEGF (b) were transfected into human islets using cationic liposome formulation, while using empty vector backbone, pCMS-EGFP (c) and nontransfected islets (d) as controls. Left, relative gene expression levels from these vectors at optimized transfection conditions. The differences in gene expression were attributed to different promoter elements. Right, transfection of human islets at different MOI of adenovirus encoding hVEGF at optimized conditions. Higher and dose-dependent gene expression is observed from adenoviral vectors. Redrawn from Narang et al. (2004), with kind permission of Springer Science and Business Media, and Cheng et al. (2004).

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analysis of kidneys isolated from these mice (Fig. 15). The functionality of the graft was confirmed by staining for human insulin, whereas stains for human von Willebrand factor and mouse CD-31 indicated vascularization originating from the expansion of human and mouse endothelial cells, respectively. Although neovascularization was observed, the gene expression levels from the islets remained low. In further studies, we have attempted to improve the gene transfer efficiency to islets using a novel lipid that contains methylsulfonic acid in the cationic headgroup region, which is expected to promote hydrogen bonding with the pDNA, resulting in

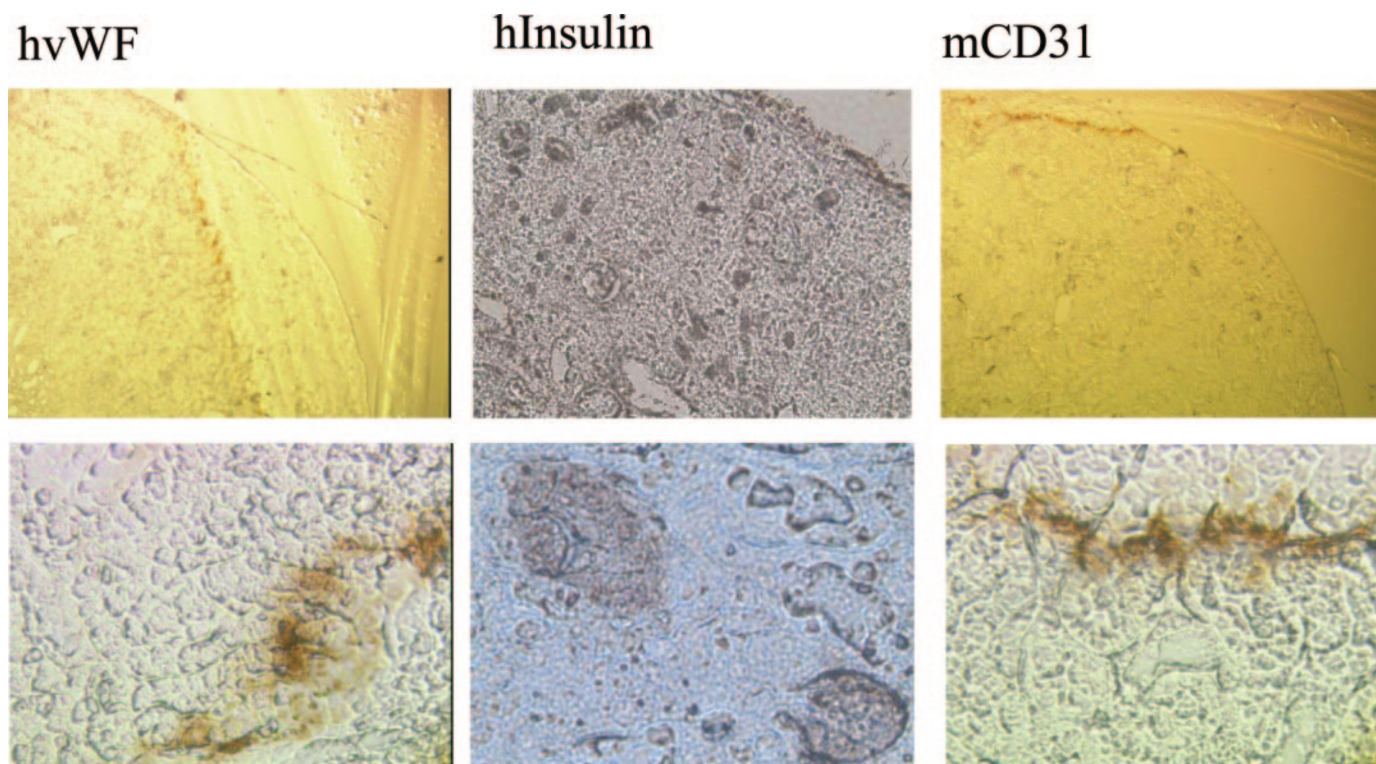

FIG. 15. Immunohistochemical analysis of mouse kidney containing transplanted islets. Kidney sections were stained with antibodies specific to human von Willebrand factor, an early marker for angiogenesis (A), human insulin (B), and mouse CD31 or platelet endothelial cell adhesion molecule (PECAM)-1, a marker of mice endothelial cells (C). Revascularization of human islets transplanted in mice proceeds with the involvement of both human and mouse endothelial cells. Reproduced from Narang et al. (2004) with kind permission of Springer Science and Business Media.

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higher lipid/DNA interactions (Narang et al., 2005). The transfection efficiency with this lipid was higher than with Lipofectamine in intact human pancreatic islets using a luciferase gene expression plasmid. The efficiency, however, was still much lower than that achieved with the Adv vectors.

Some investigators have attempted to increase the transfection efficiency of nonviral gene transfer by dispersing the islets into individual cells. For example, Saldeen et al. (1996) transfected human, mouse, rat, and fetal porcine islet cells with nonviral [calcium phosphate precipitation, Lipofectin (monocationic liposome), and Lipofectamine (polycationic liposome)], Adv, and Adv-PLL DNA complexes with chloramphenicol acetyl transferase and β-galactosidase expression vectors. They observed low transfection efficiency using nonviral vectors. However, upon dispersion of cells using trypsin treatment, approximately 50% of the cells got transfected (Saldeen et al., 1996). This higher transfection efficiency in dispersed islets is attributable to the increased surface area per cell and to the uniform exposure of all cells of islets to the transfection reagent. In normal islet morphology, the cells of the inner core of islets are not exposed to the transfection reagent as well as the surface cells, due to the diffusive barrier and the collapse of islet vasculature (Benhamou et al., 1996, 1997; Mahato et al., 2003). Furthermore, in transfection studies, the authors used high lipid and DNA dose which are toxic to cells (Mahato et al., 2003).

Nonviral transfection of islets involves an interplay of various formulation variables that must be carefully optimized to enhance transfection efficiency while minimizing toxicity. These include lipid/DNA charge ratio, cationic lipid/colipid molar ratio, cationic lipid and colipid structure, concentration of carrier/vector complexes in the final transfection medium, absolute amount of pDNA and cationic lipid per islet, volume of the transfection medium, presence of serum, and duration of incubation of islets with the complexes. Many of the issues related to these variables have been addressed in our previous publications (Mahato et al., 1998; Benns et al., 2002). In the context of pancreatic islets, the transfection efficiency and toxicity of lipid-based transfection reagents (Lipofectamine, 1,2-dioleoyl-3-trimethylammonium-propane, and 1,3-Di-Oleoyloxy-2-(6-Carboxy-spermyl)-propylamide) was studied in intact islets using a β-galactosidase expression plasmid at 12:1 liposome/pDNA ratio (Lakey et al., 2001b). Transfection efficiency was found to vary with the ratio of cationic lipid to DNA, the incubation time of complexes with the islets, and the type of cationic lipid used (Lakey et al., 2001b).

The major limitation of nonviral gene transfer methods is the low transfection efficiency and transient gene expression. The poor transfection efficiency of these vectors in islets results because the islets are a compact cluster of ~1000 nondividing cells; thus, they maintain their nuclear membrane. The nonviral vector/plasmid complexes have limited permeability to the core cells of the islets due to their large size. In addition, because plasmid DNA must enter the nucleus for transcription, transgene expression is cell cycle-dependent, with enhanced permeation during mitosis when the nuclear membrane dissolves (Brunner et al., 2000). Cationic lipid (e.g., Lipofectin and Lipofectamine) and polymer (e.g., polyethyleneimine)-based carriers, therefore, poorly transfect intact mouse, rat, and porcine islets and are toxic at high doses (Saldeen et al., 1996; Benhamou et al., 1997; Lakey et al., 2001b).

Although we and others have shown the feasibility of transfection of islets with nonviral vectors (Kenmochi et al., 1998; Lakey et al., 2001b; Mahato et al., 2003; Narang et al., 2004, 2005), transfection efficiency remains low. Further efforts are needed to investigate the role of plasmid vector and gene carrier design considerations to improve transfection efficiency. Interested readers are referred to reviews on these topics in our recently published books (Mahato and Kim, 2002; Mahato, 2005). In contrast, as described below, replication-deficient Adv vectors efficiently transfect both dividing and nondividing cells including intact islets (Csete et al., 1995; Muruve et al., 1997). Hence, there has been a shift of focus toward higher transfecting Adv vectors, while attempting to solve problems related to their toxicity and immunogenicity.

B. Viral Gene Delivery

Various recombinant viral vectors may be used for ex vivo and in vivo gene transfer including Adv (Giannoukakis et al., 1999b; Cheng et al., 2004), adeno-associated virus (Flotte et al., 2001; Kapturczak et al., 2002), retroviral (Leibowitz et al., 1999), lentiviral (Gallichan et al., 1998; Giannoukakis et al., 1999a), and herpes simplex virus (Liu et al., 1996; Rabinovitch et al., 1999). Each of these vectors has specific characteristics that provide selection features for specific applications. For example, whereas Adv vectors provide high transfection efficiency, the gene expression is only transient. Retroviral vectors, on the other hand, have relatively lower transfection efficiency, but there is long-lasting gene expression due to vector integration in the host chromosome. This, however, leads to oncogenic potential of these vectors, which increases their safety risk. Interested readers are referred to reviews discussing the merits and disadvantages of various viral vectors (Robbins and Ghivizzani, 1998; El-Aneed, 2004; Tomanin and Scarpa, 2004).

Transfection efficiency of replication-deficient Adv vectors is 90 to 95%, which exceeds that of retroviral and lentiviral vectors (Leibowitz et al., 1999). Some reports, however, indicate that lentiviral vectors transfect islets at similar efficiency compared with Advs (Giannoukakis et al., 1999a). In addition, islet cell dispersion increases the gene transfer efficacy for most gene transfer vectors

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including retroviruses (Leibowitz et al., 1999). This, however, would not be preferred because of the loss of islet integrity and cell-to-cell interactions that are important for normal islet function.

Adv vectors can be produced in high titers, and there is no risk of insertional mutagenesis as their genomes are not integrated into the host chromosomes. Several factors pertinent to the clinical scenario of islet transplantation limit the disadvantages of Adv vectors. Although the host immune response against Adv vectors is well known at high multiplicities of infection (MOI) involving direct administration of Adv for in vivo applications, this may not be significant for ex vivo gene delivery to islets, where islets are transfected in Petri dishes and washed of viral particles prior to transplantation. Most of the Adv genomes are, therefore, inside the cells at the time of infusion of transfected islets into the islet transplant recipients. In addition, in recent studies, the direct local delivery of low and intermediate dose of Adv vectors in humans indicated that these vectors are well tolerated even up to the dose of $\leq 10^{11}$ particles (Harvey et al., 2002). Furthermore, the recombinant Advs can be screened by standard procedures (Hehir et al., 1996) to rule out contamination with replication competent adenovirus (RCAs) to reduce the risk of nontarget cell infection in the host and a rigorous immune response. This ensures that the administered Advs are confined to the transplanted islets and not spread out to other cells and tissues, thus reducing the likelihood and extent of nonspecific inflammation and specific immune reactions to Adv vectors.

In our studies with intact human islets, we observed low transfection efficiencies with nonviral transfection methods, even after vector backbone and gene carrier modifications. Our subsequent use of Adv vector-based islet transfection resulted in significantly higher gene expression profiles, which were confirmed at both mRNA and protein levels (Fig. 14B). Gene expression from Adv vectors was studied as a function of viral dose (in terms of MOI per IE) and duration of incubation of islets after transfection. We observed a dose-dependent increase in islet transfection and toxicity. The dose of 500 MOI was selected as optimum for good gene expression levels with minimal toxicity to the islets. Thus, although VEGF protein secretion in the media from nonviral vectors was ~6 ng/1000 IE in 10 days (Fig. 14A), the expression from 500 MOI of Adv-transfected islets was approximately 10 times higher (~60 ng/1000 IE) (Fig. 14B).

Gene expression was Adv dose-dependent, and the transfection procedure did not cause significant toxicity to the islets (Cheng et al., 2004). Whereas the humoral immune response is directed to capsid proteins after initial administration and significantly reduces efficiency of gene expression following repetitive gene delivery, the cellular immune response is attributable to the viral structural proteins formed inside transduced cells by the expression of late gene products (Liu and Muruve, 2003). Although the innate immune responses are weak in vivo because of the absence of free Adv particles in the islet infusion, adaptive immune responses directed to either the residual expression of viral genes from the recombinant vector and/or transgene products in the islets may be significant. For the latter, the "self"-recognition of transgene products is important to prevent transgene-directed immune responses. Hence, only islet graft recipient species-specific gene sequences should be used in generating recombinant Adv vectors. Furthermore, two kinds of approaches are used to combat the first two safety concerns: 1) vector backbone modification to minimize the residual expression of viral genes and 2) virus surface modification to reduce host responses against free viral particles.

1. Vector Backbone Modification. Adv genome consists of a single, linear, double-stranded DNA molecule of ~35 kb, of which ~30 kb can be replaced with foreign DNA (Smith, 1995). It has short terminal repeat sequences that contain one origin of replication each. The genome also has *cis*-acting packaging sequence. The viral chromosome contains various transcriptional units that are transcribed by RNA polymerase II at different times after infection. Their roles are summarized in Table 3 (Shenk, 1996). These genes are transcribed in a chronological sequence, as indicated in the table. Arrangement of viral genes on the genome has been suggested to serve a "timing" function. Viral core has an organized, compact structure that gets converted to a more open structure as the early phase of infection. It has been proposed that the RNA polymerase initially interacts with promoters at the ends of the chromosome, and the transcription of terminal units drives further opening of the compact structure. Thus, insertion of a strong transcription termination sequence between E1A and E1B inhibited activation of the E1B unit (Shenk, 1996).

The initial, so-called "first-generation" Adv vectors involved deletion of the E1 gene, in whose place was inserted the transgene of interest. These ΔE1 vectors were shown to deliver transgenes to a wide variety of cells with high efficiency (Wang and Finer, 1996). The safety concerns with these vectors include the host immune response, a direct cytopathic effect on transduced cells, generation of RCAs, and reduced persistence of transgene expression (Wang and Finer, 1996). Adv DNA replication is required for expression of late gene products and is regulated by many viral proteins encoded by E1, E2, and E4 gene regions (Wang and Finer, 1996). It was observed that ΔE1 vectors had leaky viral DNA replication, which was followed by low-level viral late protein production. At high MOI, the E1 region became dispensable for replication, which could be related to the expression of host gene products with E1A-like activity or to the proliferative state of cells. Accumulation of cytotoxic late gene products also leads to a direct cytopathic effect

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TABLE 3
Viral genome organization

Viral DNA is transcribed from both strands with the so-called 'rightward' reading strand encoding for E1A, E1B, IX, major late, virus-associated RNA, and E3 units, whereas the 'leftward' reading strand encodes E4, E2, and IVa2 units. Either Orf 3 or Orf 6 is sufficient to provide E4 functions for normal viral lytic cycle. Orf 6 protein forms a physical and functional complex with E1B protein, which mediates shutoff of host protein synthesis and efficient transport of late mRNAs from the nucleus to the cytoplasm and maintains their stability. Orf 3 has a redundant function and acts in parallel with the complex to permit normal viral DNA replication. Deletion of both Orf 3 and Orf 6 is lethal to Advs (Wang and Finer, 1996).

Early transcription units	
E1	
E1A	Encodes two proteins (by alternative splicing) that activate transcription and induce host cell to enter S phase of the cell cycle
E1B	Encodes two proteins that cooperate with E1A to products to induce cell growth; also blocks host mRNA transport and stimulates viral mRNA transport
E2	E2 encodes three proteins, all of which function directly in DNA replication
E2A	72-kDa DNA binding protein
E2B	Encodes DNA polymerase terminal protein
E3	Modulates immune response of infected cells
E4	E4 proteins mediate transcriptional regulation, mRNA transport, and as DNA replication
Orf 6 and 7	Bind transcription factor E2F, leading to activation of E2 region of Adv and upward expression of replication proteins; only Orf 6 essential for viral growth in tissue culture
Orf 3	Increases stability of late viral transcripts
Orf 4	Indirectly down-regulates E1A expression, leading to reduction in viral DNA accumulation unless Orf 3 or Orf 6 counteracts its effect
Delayed early transcription units	
IX	
IVa2	
Major late transcription unit	Driven by major late promoter; late transcripts encode virion's structural proteins; virus-associated RNAs are required for translation of late viral transcripts
L2	Encodes penton
L3	Encodes hexon
L5	Encodes fiber

on transduced cells in addition to the triggering of host cellular immune response. These lead to local inflammation and destruction of transduced cells and result in limited persistence of transgene expression (Wang and Finer, 1996). For these reasons, additional deletions were desired to reduce the production of late gene products by crippling the ability of virus to replicate and make an early-to-late phase transition in its life cycle.

Introduction of a temperature-sensitive mutation in the E2A region was expected to cripple the ability of Adv to replicate its DNA since the E2A region encodes a single-stranded DNA-binding protein, with specific affinity for single-stranded Adv DNA (Lusky et al., 1998), that plays an essential role in DNA synthesis and chain elongation during replication by protecting the single strands generated during replication from nuclease digestion. DNA-binding protein also plays a central role in the activation of major late promoter during the late phase of infection (Lusky et al., 1998). Additional mutations of the E2A region reduced viral late protein production and inflammation and enhanced persistence of transgene expression from 14 to 70 days (compared with ΔE1 Adv) at nonpermissive temperatures (40.5°C) (Engelhardt et al., 1994). This difference, however, was not observed in animal models at permissive temperatures (≤37°C) (Fang et al., 1996). In a subsequent study, deletion of the E2A region was shown to reduce viral protein production as well as accumulation of infectious virions without affecting gene transfer compared with first-generation vectors (Gorziglia et al., 1996). Deletion of the E2B region, which encodes viral DNA polymerase (Δpol vector), has also been shown to significantly reduce Adv late gene expression (Amalfitano et al., 1998). These vectors (ΔE1/ΔE2A or ΔE2B) with an additional deletion have been termed "second-generation" Advs.

The Adv E4 region contains seven open reading frames, which encode various regulatory proteins (Table 3), whose defects were observed to cause inhibition of viral DNA synthesis, reduction of late gene expression, instability of late mRNAs, and failure of host T-cell shutoff (Wang and Finer, 1996). Hence, deletion of the E4 region in addition to E1 cripples viral late protein production with the desired improvement in safety and efficacy profiles. These vectors (ΔE1/ΔE4) have also been termed second generation, signifying an additional crippling deletion in the Adv genome.

The second generation Advs also reduce the danger of RCA generation during Adv production, because two recombination events would be required. The frequency of homologous recombination-mediated by an Adv vector in nonpermissive cells (10^{-5}–10^{-6}) (Wang and Taylor, 1993; Fujita et al., 1995) is further reduced to $\sim10^{-11}$ (Wang and Finer, 1996). Deletion of the E4 region is expected to further improve the safety profile of Adv vectors because the E4 Orf 6 protein is oncogenic (Dobner et al., 1996; Moore et al., 1996). Development of a complementing cell line for Adv production, however, was a daunting task because E4 proteins are cytotoxic, and their overexpression leads to cell death (Wang and Finer, 1996). Overproduction of E4 proteins is induced

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by the E1A gene product 13S in trans, both of which needed to be expressed in the same cell line. This problem was overcome by replacing the E4 promoter with the mouse α-inhibin promoter to generate the 293-E4 cell line (Wang et al., 1995a; Wang and Finer, 1996).

E3 region genes are not expressed in the absence of induction from E1A protein and are not required for Adv replication (Gorziglia et al., 1996). This deletion has been used (in ΔE1 and ΔE3 vectors) primarily to allow for the insertion of large transgenes, since the E3 region follows the E1 region in the same DNA strand. Hence, the E3 region deletion in Advs has been used in both first- and second-generation Advs and is not considered a criterion for "generational classification" of Advs by most researchers.

The first-generation (E1 and E3 deleted) Adv vectors are known to induce cellular and humoral immune responses in vivo at high MOI due to residual expression of viral proteins. The use of second generation (E1, E3, and E4 deleted) Adv vectors has been reported to cause very little immune response because of the additional deletions of viral genes (Yang et al., 1994b; Wang and Finer, 1996; Danthinne and Imperiale, 2000). The result is improved duration of gene expression and reduced cell-mediated immune responses for second generation Adv vectors (Liu and Muruve, 2003) However, the production of E1-, E3-, and E4-deleted second generation Advs is slower, and the final viral titers are usually approximately 10-fold lower than that of E1- and E3-deleted first-generation Advs (He et al., 1998). Furthermore, although many researchers have found an improvement in the persistence, toxicity, and immunogenicity of second-generation Advs compared with first-generation vectors (Engelhardt et al., 1994; Gorziglia et al., 1996; Dedieu et al., 1997; Wang et al., 1997a; Everett et al., 2003), some groups have found them to be comparable (Fang et al., 1996; Morral et al., 1997; Wen et al., 2000). These observations are attributed to a possibly minor role of cellular immune response to the progressive elimination of the viral genome (Lusky et al., 1998) and to the existence of an interplay between anti-Adv and antitransgene immune responses (Michou et al., 1997; Morral et al., 1997; Lusky et al., 1998). Hence, whereas for most of the data showing a difference in transgene persistence bacterial β-galactosidase was used as a model gene, Lusky et al. (1998) did not find a difference in the persistence and immunogenicity of ΔE1, ΔE1/ΔE2A, and ΔE1/ΔE4 deleted vectors that lacked a transgene. Some reports also found the presence of intact E4 to be important in preventing apoptotic cell death in culture conditions (Jornot et al., 2001).

Although the deletion of additional regions of the Adv genome has been shown to reduce immunogenicity, toxicity, and inflammation and to improve safety and persistence in some cases, there also have been conflicting reports on such improvements. It seems that the extent of such improvement would vary with the gene candidate, disease target, and the relative proportion of the role cellular immunity plays in determining the immunogenic and harmful reactions as well as transgene persistence. Other advantages of deleting the Adv genome, e.g., reduced recombination frequency and deletion of regions with known oncogenic potential, remain unchallenged. Increasing deletion of the Adv genome also makes vector production more difficult with the requirement of complementing cell lines and low viral titers obtained.

To further improve the safety profile by vector backbone modification, gutless, or helper-dependent, Adv vectors were produced. These Advs have all the Adv genes deleted and hence provide an excellent safety profile. These vectors contain only the inverted terminal repeats and a packaging sequence around the transgene (Chen et al., 1997). However, they require a helper virus during their production (to provide all the necessary viral genes in trans) and complete purification of these viruses from helper virus has not been successful until now—a major limitation for and concern with their application.

ΔE1/ΔE2A/ΔE3 vectors were termed "third generation" Adv vectors, whereas ΔE1/ΔE2A/ΔE3/ΔE4 vectors were called "fourth generation" (Andrews et al., 2001). A practical problem with higher generations of Adv vectors with additionally deleted genome components is in their production, which becomes increasingly less efficient, and the achievable titers of Advs get reduced. Thus, although deletion of both E2A and E4 additional regions is expected to improve the safety and persistence of vectors, the problem of efficient production is even more severe with these vectors. Thus, the production of ΔE1/ΔE2A/ΔE3/ΔE4 vector could not be scaled up for in vivo studies, whereas similar vectors that retained Orf 3 of the E4 region could be mass produced (Andrews et al., 2001). Thus, although first-generation (ΔE1 and ΔE1 and ΔE3) vectors continue to be used the most in preclinical investigational studies, the second-generation (ΔE1 and ΔE2a) vectors are preferred for their improved safety profile.

2. Surface Modification of Viral Vectors. Viral and nonviral gene transfer vectors have contrasting properties in their gene transfer mechanisms. Whereas the viral vectors present an excellent profile of endosomal release, cytoplasmic stability, and nuclear entry, their attachment and internalization are surface coxsackievirus and adenovirus receptor (CAR)-dependent. Lipid-based nonviral vectors, on the other hand, have excellent attachment and internalization but poor endosomal escape, cytoplasmic stability, and nuclear entry properties. Furthermore, although viral vectors are potent immunogens, nonviral vectors have poor immunogenicity. A rational combination of both viral and nonviral vector components, thus, has promise to overcome the limitations of viral vector-mediated transfection. This may be achieved by viral surface coating with lipid (Gregory et

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al., 2003); administration of viruses in liposomes (Ohmori et al., 2005); or Adv surface conjugation of ligands, spacers (Eto et al., 2005), and polymers (Fisher et al., 2001).

Surface modification of Advs has been attempted toward various ends including cell specific targeting, change of tropism, and modification of the surface properties and antigenicity of Advs. For example, Kawakami et al. (2003) have modified the tropism of Advs by changing the surface serotype 5 knob with serotype 3 knob protein to target tumors with low surface expression of the Adv 5 receptor, CAR. Improving the transfection of Advs to tissues that display low-level expression of CAR has also been targeted by Adv fiber modification with integrin-binding RGD peptide and PLL motifs to achieve Adv infection via CAR-independent pathways (Wu et al., 2002). Another approach for the infection of CAR-deficient cell types is the capsid surface modification of protein IX to incorporate PLL to target heparan sulfate receptors (Dmitriev et al., 2002). These strategies, however, have not been well investigated for ex vivo transfection of islets prior to transplantation. This may be due to the fact that islets are rich in CAR and show high transfection efficiency with unmodified Advs.

PEGylation of Adv has been shown to protect the vectors from pre-existing and adaptive immune responses by reducing their interactions with immune cells (O'Riordan et al., 1999; Croyle et al., 2002; Eto et al., 2005; Mok et al., 2005). Mok et al. (2005) studied PEG-modified first-generation and gutless Adv vectors for their transfection efficiency and immunogenicity in vitro and in vivo. The authors observed reduced transfection efficiency of PEGylated vectors in vitro due to reduced vector-cell interactions and possible masking of the Adv knob domain that interacts with CAR. The transfection efficiency, however, was similar to that of unmodified Adv in vivo, possibly due to Adv interactions with heparin sulfate, proteoglycan, and integrin receptors (Mok et al., 2005). In terms of immunogenicity, the authors observed a reduction of IL-6 production by 50 to 70% compared with native Advs and a corresponding reduction of vector uptake by macrophages (in vitro) and Kupffer cells (in vivo). These results demonstrate the applicability of modified vectors in vivo. However, in the case of ex vivo islet transfection, the utility of PEGylated Adv vectors may be offset by lower in vitro transfection efficiency because of islets being clusters of ~1000 cells. The lowering of transfection efficiency of PEGylated Advs can also be overcome by attachment of transfection-enhancing ligands on the tip of PEG, e.g., RGD peptide. Using this strategy, Eto et al. (2005) produced RGD-PEG-Advs that showed 200-fold higher gene expression than PEG-Adv and similar to that of RGD-Adv. A reduction in immunogenicity combined with an exceedingly low dose of Adv possibly administered with islet transplantation holds the promise to significantly overcome the safety concerns with the use of Advs for ex vivo gene transfer to islets.

C. Antisense Oligonucleotides and RNA Interference for Gene Silencing

Silencing of genes that encode proinflammatory cytokines or that are intermediaries in the apoptotic pathway of islets can be useful for improving the prospects of islet transplantation. Such genes include the proinflammatory cytokines secreted by the resident islet macrophages, e.g., IL-1β, TNF-α, and IFN-γ, that participate in apoptotic cell death. A central biochemical pathway that leads to islet destruction, activated by various stimuli, is the induction of the inducible NO synthase (iNOS) gene and release of toxic quantities of NO in the immediate vicinity of β-cells within the islets, leading to loss of function and apoptosis of islets (Stevens et al., 1996). Cytokines such as IL-1β, TNF-α, and IFN-γ released by various sources stimulate NO production within the islets, which leads to loss of islet function and cell death by apoptosis (Fig. 15). Although islet macrophage depletion by culture at 24°C can attenuate the Fas-mediated pathway (Arnush et al., 1998b; Bottino et al., 1998), cytokine-mediated cell death can be prevented by inhibiting NO production by islets (Brandhorst et al., 2001a, 2001b; James, 2001). Silencing this central pathway to islet destruction may significantly improve the survival and function of islets after transplantation.

Gene silencing can be achieved at the translational level using either antisense oligonucleotides (ODNs) or RNA interference (RNAi). Furthermore, both antisense ODNs and small interfering RNAs (siRNAs) are 8 to 16 kDa in molecular mass, which enables them to transfect islets more efficiently than plasmid DNA. RNAi is a novel mechanism whereby small RNA nucleotides cleave target mRNA in a sequence-specific manner (Caplen and Mousses, 2003; Schutze, 2004). Several recent reviews provide insights into this novel mechanism (Bagasra and Prilliman, 2004; Mello and Conte, 2004; Stevenson, 2004; Matzke and Birchler, 2005; Sontheimer, 2005; Zhang et al., 2005), whose application to islets has been only recent and limited (Ehses et al., 2003; Abderrahmani et al., 2004; Bain et al., 2004; da Silva Xavier et al., 2004; Darville et al., 2004; Diaferia et al., 2004; Knoch et al., 2004; Nakajima-Nagata et al., 2004; Narita et al., 2004; Welsh et al., 2004; Bradley et al., 2005; Hagerkvist et al., 2005). Use of siRNAs for gene silencing using the RNAi technology involves cell treatment with small double-stranded oligoribonucleotides, which are taken up by the cells and degrade mRNA in a sequence-specific manner. Although being very effective and widely applied, the siRNA technology requires careful consideration in the design of double-stranded interfering RNA molecules (Mahato et al., 2005), e.g., the duplex sequence should be from the exposed regions of the RNA tertiary structure, and multi-

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ple RNA duplexes are often combined to achieve effective silencing.

Although some authors have elaborated on the efficiency of RNAi-mediated gene silencing in islets (Bain et al., 2004; Bradley et al., 2005; Hagerkvist et al., 2005), most work has been focused on elaborating the role of specific genes and intracellular signaling pathways (Ehses et al., 2003; Waselle et al., 2003; Abderrahmani et al., 2004; Cheviet et al., 2004; da Silva Xavier et al., 2004; Darville et al., 2004; Diaferia et al., 2004; Iezzi et al., 2004; Knoch et al., 2004; Nakajima-Nagata et al., 2004; Narita et al., 2004; Welsh et al., 2004; Schisler et al., 2005). RNA interference can be mediated by two kinds of vectors: 1) siRNA molecules, which are approximately 20 nucleotide double-stranded RNAs, delivered by lipid-mediated transfection and 2) efficiently transfecting Adv vectors that produce small hairpin RNA molecules in situ that get cleaved to generate the desired siRNA molecules. In either case, the siRNA molecules silence the target genes in a sequence-specific manner. Although liposome-mediated delivery of siRNA molecules is the most widely used RNAi technique, Adv transfection of siRNA encoding gene sequences may be particularly efficacious in the case of islets because of the high transfection efficiency of Advs and the rapidity with which Adv constructs can be prepared using recombinant DNA technology. Thus, Bain et al. (2004) demonstrated effective silencing of GLUT2 glucose receptor and glucokinase in intact rat islets using Adv vectors expressing sequence-specific siRNAs.

SiRNAs have been explored for improving the prospect of islet isolation by reducing ischemia-induced apoptotic loss of cells during procurement, preservation, and islet isolation from the pancreas. Using fluorescent-labeled siRNAs, Bradley et al. (2005) recently demonstrated that siRNAs can be delivered to the islets by both intravenous tail vein injection in mice and by perfusion of isolated pancreas before islet isolation. This technique opens the possibility of interventions prior to islet isolation to improve the quality of isolated islets.

Reports on the therapeutic application of these strategies to improve the outcome of islet transplantation, however, have been limited. One promising area of application for these strategies is the targeted silencing of iNOS gene, which is involved in and central to cytokine-mediated toxicity to the islets (Fig. 13) (Rabinovitch et al., 1990; Dunger et al., 1996; Arnush et al., 1998a; Titus et al., 2000). Guo et al. (2002) explored the antisense oligonucleotide strategy to block the iNOS pathway in rat islets by targeting the NADPH-producing enzyme glucose-6-phosphate dehydrogenase (G6PD) (Guo et al., 2002). NADPH is an obligatory cosubstrate for iNOS production of NO. The authors observed a significant reduction in IL-1β-induced NO production by silencing of G6PD gene expression using an antisense ODN targeting G6PD (Guo et al., 2002).

Although most of the applications of antisense ODNs are also focused on elaborating intracellular signaling pathways in islets (Kulkarni et al., 1996; Kajimoto et al., 1997; Meng et al., 1997), they have also been used to aid in the protection of islets against immune-mediated destruction. Thus, Katz et al. (2000) have explored the benefit of silencing ICAM-1 and its ligand, LFA-1, in mice pancreatic islet allotransplants by post-transplantation treatment of recipients with phosphorothioate ODNs. The authors observed that ICAM-1/LFA-1 blockade improved islet function and reduced inflammation compared with untreated controls (Katz et al., 2000).

Both antisense ODNs and RNAi strategies provide promising tools for silencing target genes in intact islets, but their applications currently are focused primarily on investigating of signaling pathways in islets. A significant advantage of these techniques is simultaneous silencing of a family of genes, e.g., proinflammatory cytokines, by selecting target sequences in the overlapping region of various gene products. Increasing applications of these strategies to therapeutic down-regulation of gene expression either alone or in combination with up-regulated expression of targeted survival genes is expected to be tremendously beneficial to improving the outcome of islet transplantation.

VI. Concluding Remarks

Islet transplantation is a potential treatment option for patients with type I diabetes, allowing stable glucose homeostasis without exogenous insulin administration. Although tremendous progress has already been made in the isolation and culture of human islets, a large number of islets is needed to obtain insulin independence in clinical islet transplantation, requiring two to four cadaveric pancreases. Thus, much higher numbers of islets are required than are currently available. In addition to prevention of β-cell death post-transplantation to reduce the number of islets required per patient, islets from different species (xenotransplantation), stem cells (neogenesis), or pre-existing islets (regeneration therapy) are being evaluated to overcome the shortage. Preventing immune destruction of transplanted islets currently necessitates lifelong immunosuppression of the patient using strong immunosuppressive agents, limiting the application of islet transplantation in clinical practice. Graft-specific immune tolerance induction in the host and microencapsulation of islets to protect the graft from host immune cells are being explored to possibly bypass the need for immunosuppression.

Nucleic acid-based therapeutics have a unique advantage in the scenario of islet transplantation because islet culture is an essential part of the procedure, and it can easily be combined with other approaches to achieve synergistic benefits. Ex vivo nucleic acid therapy offers the advantage of genetically engineering islets prior to transplantation. Although the transfection efficiency of

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nonviral systems, such as cationic lipids and polymers, into human islets remains low and toxic at high doses, several viral vectors, including Adv, lentiviral, and adeno-associated viral vectors, have shown promise. The host immune reaction against Adv vectors is well known when they are administered at high MOI; however, this should not be a significant problem because islets are transfected ex vivo and most unabsorbed virus particles are washed off before transplantation. Moreover, the direct administration of low and intermediate doses ($\sim 10^{11}$ particles) of Adv vectors in humans is well tolerated. Current research indicates the need for applying more than one strategy simultaneously to achieve synergistic benefits in overcoming the many barriers limiting islet transplantation.

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