Prospective and Challenges of Islet Transplantation for the Therapy of Autoimmune Diabetes

A.N. Balamurugan, PhD,† Rita Bottino, PhD,† Nick Giannoukakis, PhD,†‡ and Cynthia Smetanka, MD**

Abstract: Pancreatic islet cell transplantation is an attractive treatment of type 1 diabetes (T1D). The success enhanced by the Edmonton protocol has fostered phenomenal progress in the field of clinical islet transplantation in the past 5 years, with 1-year rates of insulin independence after transplantation near 80%. Long-term function of the transplanted islets, however, even under the Edmonton protocol, seems difficult to accomplish, with only 10% of patients maintaining insulin independence 5 years after transplantation. These results differ from the higher metabolic performance achieved by whole pancreas allotransplantation, and autologous islet cell transplantation, and form the basis for a limited applicability of islet allografts to selected adult patients. Candidate problems in islet allotransplantation deal with alloimmunity, autoimmunity, and the need for larger islet cell masses. Employment of animal islets and stem cells, as alternative sources of insulin production, will be considered to face the problem of human tissue shortage. Emerging evidence of the ability to reestablish endogenous insulin production in the pancreas even after the diabetic damage occurs envisions the exogenous supplementation of islets to patients also as a temporary therapeutic aid, useful to buy time toward a possible self-healing process of the pancreatic islets. All together, islet cell transplantation is moving forward.

Key Words: human islet transplantation, autoimmunity, immunosuppression, islet isolation

(*Pancreas* 2006;32:231–243)

Type 1 diabetes (T1D), a chronic metabolic disorder that currently afflicts approximately 5 million individuals in the world, results from autoimmune-mediated destruction of insulin-secreting beta-cells in the islets of Langerhans of the pancreas.[1,2] Deficient endogenous insulin production places a substantial burden upon a patient's quality of life, especially children and adolescents that are the most frequent target population of autoimmune diabetes.[3] Despite exogenous insulin therapy, normal physiological glycemic control can only be achieved by beta-cell replacement, thus restoring in vivo insulin secretion from the beta-cells of the islets of Langerhans. Regular or even intensive exogenous insulin treatment does not warrant normal glucose levels at all times. Maintaining stable glucose levels is highly important for preventing the development of secondary complications. The mortality and morbidity related to poor blood glucose control have been studied by the Diabetes Control and Complication Trial (DCCT),[4,5] the Epidemiology of Diabetes Interventions and Complications (EDIC),[6] and the UK prospective diabetes study (UKPDS).[7] These reports demonstrate the need for maintaining normal physiological glycemic levels to prevent or delay the progression of macrovascular and microvascular complications.

Beta cell replacement can be achieved by either whole organ pancreas transplantation or isolated islet cell transplantation. Transplantation of the whole pancreas is a well-consolidated procedure, performed in more than 20,000 patients,[8] that requires major surgery and often presents complications related to the excessive exocrine drainage of the implanted pancreas. Although there are some unquestionable solid advantages achieved by whole pancreas transplantation, like long-term stable normoglycemia, the procedure entails considerable risks to the recipient.[9,10] Most pancreatic transplants are carried out in association with, or following, kidney transplantation in those diabetic recipients that already suffer from renal failure, a typical complication of diabetes. These patients can significantly benefit, considering the double burdens associated with diabetes and kidney disease, and certainly improve their life style sufficiently to justify the risks of surgery and life-long immunosuppression.[11] The advantage of islet cell transplantation stems from its relatively simple administration route that does not require major surgical procedures, it can be performed on an outpatient basis under local anesthesia, with the supervision of a trained interventional radiologist, and can be repeated several times without major discomfort to the patient.[12]

Approximately 3500 patients worldwide have received a pancreas transplant over the last 4 years (2500 as simultaneous kidney-pancreas, 712 pancreas after kidney and 278 solitary pancreas) according to the International Pancreas Transplant registry (IPTR),[8,13] versus 470 islet allografts within the same period,[14] mostly subsequently to the introduction of the Edmonton protocol. The American Diabetes Association considers pancreas transplantation for diabetic patients with imminent or established end-stage renal disease who have had or plan to have a kidney transplant.[15] However, in the absence of indications for kidney transplantation, pancreas transplantation should be considered only when severe

Received for publication October 10, 2005; accepted November 22, 2005.
From the *Thomas E. Starzl Transplantation Institute, Department of Surgery, University of Pittsburgh, Pittsburgh, PA; †Division of Immunogenetics, Department of Pediatrics, University of Pittsburgh School of Medicine, Children's Hospital of Pittsburgh, Pittsburgh, PA; and ‡Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA.

Reprints: A.N. Balamurugan, PhD, Department of Surgery, Thomas E. Starzl Transplantation Institute, University of Pittsburgh, Pittsburgh, PA 15261 (e-mail: isletologist@hotmail.com).

metabolic complications, such as hypoglycemia, hyperglycemia, and ketoacidosis, occur with a frequency that poses a threat to the patient. Patients with vascular complications are not considered for whole pancreas transplantation. Islet transplantation, also because of the risks associated with systemic immunosuppression, is considered an experimental procedure and it is restricted to adult individuals with "brittle diabetes" who do not suffer from secondary complications.[16,17]

Islet transplantation has been considered a potentially suitable therapy for T1D for years, especially after accumulating evidence in rodents that viable and physiologically functioning islets could be extracted from the pancreas of a donor, purified from the exocrine component of the pancreas, and infused in the portal vein of a diabetes-induced rat recipient, achieving stable euglycemia.[18] Further evidence of the effectiveness of islet transplantation was accrued in the 1970s when pilot centers worldwide introduced the clinical practice of islet autotransplantation.[19,20] Surgical removal of the pancreatic organ is a drastic but necessary therapy for severe untreatable pancreatitis, trauma, or benign neoplasm.[21,22] To prevent diabetes in pancreatectomized patients, islets are obtained from the explanted pancreas and reintroduced intraportally or intraperitoneally in the same donor/recipient after elimination of most of the acinar tissue. The major problem and limit is the islet mass that can be obtained from the pancreas, considering that the organ tissue is usually fibrotic, making it difficult to get a very pure preparation of islets. Moreover, the outcome of islet autotransplantation depends on the islet yield obtained at the end of the process of excising, digesting, and purifying the pancreas. The Minnesota group, the first to introduce this technique in clinical practice, and various other groups, reported that at least 3000 islet equivalent number (IEQ)/kg are needed to ensure adequate beta-cell function, with approximately 200,000 IEQ necessary to achieve insulin independence in patients, corresponding to approximately 20–30% of the islet content of a pancreas.[23] Long-term insulin independence may even require a higher islet mass, greater than 5000 IEQ/kg. Therefore, islet mass is a predictive parameter for long-term duration of the islet autograft.

The International Islet Transplant Registry (ITR)[24,25] reported insulin independence beyond 1 year in approximately 50% of the first 240 islet autografts, regardless of the number of islets infused. When the islet number infused was ≥300,000, the success was 71% (Fig. 1A). The longest period of insulin independence after islet autotransplantation has exceeded 13 years.[26] Data clearly show improved clinical outcomes as the techniques of isolation have progressed over the past 25 years. The autotransplantation model proves the concept that islet cells separated from the exocrine tissue and implanted ectopically in the liver or the peritoneum are able to establish and maintain glucose homeostasis. Absence of rejection and autoimmunity are the 2 main reasons behind the success of such an approach. A more complex scenario, in fact, characterizes the applicability of islet transplantation between different donor/recipient individuals, namely, allotransplantation, in particular in the case where islets are most needed: recipients having autoimmune diabetes.

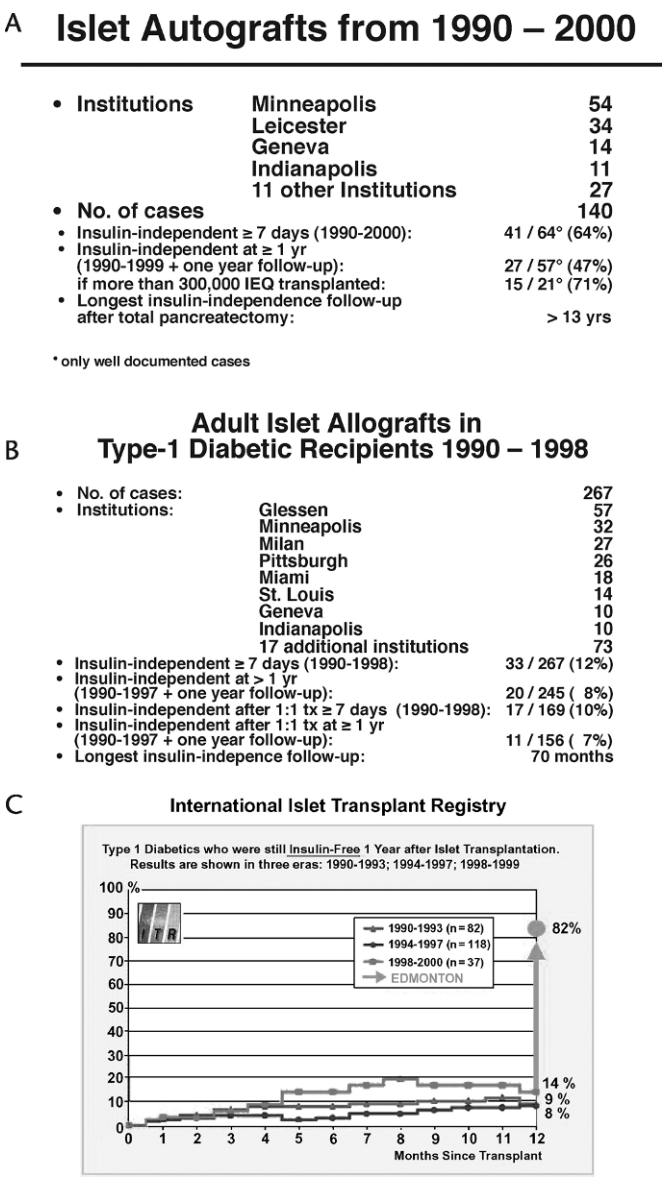

FIGURE 1. A, Data from the International Islet Transplant Registry show that an adequate islet mass (over 300,000 IEQ) infusion achieves insulin independence in 71% of cases of islet autografts. B, Data from the International Islet Transplant Registry show that among 267 cases of adult islet allotransplantation performed between 1990 and 1998, only 8% of the patients remained insulin independent for more than 1 year. C, Data from the International Islet Transplant Registry show the 1-year insulin independency rate after islet allograft increased under the Edmonton protocol.

In view of supplying islets from a donor to a diabetic human recipient, and minimizing the expected alloimmune response, it became clear that only minimal volumes of purified islets were to be transplanted. Technically, the isolation of human islets from the cadaveric pancreas in sufficient amounts to even consider allotransplantation procedures remained an elusive goal until a new method for the extraction of human islets from the pancreas of deceased

human donors was described in 1989.[27] This new approach warranted consistently high numbers of purified and viable human islets.[27] Moreover, despite the availability of larger islet numbers, attempts at transplanting human islets to diabetic recipient were rather unsuccessful. Exceptions came in the form of very limited studies, such as those carried out at the University of Pittsburgh in the early 1990s, where cancer patients subjected to major resective surgery received multiorgan allotransplants with the inclusion of islets, which conferred good metabolic glycemic control without the need for exogenous insulin injections.[28] To note, the recipients of multiple organs were surgically diabetic but not affected by autoimmune diabetes. A total of 445 adult islet allotransplantations were performed worldwide between 1974 and 2000, according to the Islet Transplant Registry;[25] however, when compared to the outcome of whole pancreas transplantation, the results were quite disappointing until the year 2000. Of the 267 islet allotransplantations performed from 1990 to 1998, insulin independence after 1 year was achieved in only 8% of the patients[24] (Fig. 1B).

In the year 2000, Shapiro et al[29] at the University of Alberta in Canada reported successful reversal of diabetes by pancreatic islet allotransplantation. Their study focused on the use of islet cell transplantation alone for a subgroup of T1D patients with severe hypoglycemia and uncontrolled diabetes, but no kidney disease. Their novel immunosuppressive regimen, associated with a meticulous preparation of the islets, implanted in large masses, later named the "Edmonton protocol," revolutionized the field of islet transplantation. Their results showed that their first consecutive 7 patients, became free of the need for insulin therapy.[29] This outcome was far superior to any result ever obtained with islet allografts to T1D patients until then.[30] Their protocol adapted all the current improved techniques for pancreas procurement and isolation. The major novel approach was to transplant an adequate islet mass through repeated islet administrations on a corticosteroid sparing-based immunosuppressive regimen. The immunosuppressive regimen used in their islet allograft trials renewed interest in islet transplantation for the cure of diabetes and an increasing number of centers entered the field and applied the protocol. The main features of it include harvesting the pancreas before multiorgan retrieval, avoidance of prolonged cold storage of the pancreas (<8 hours), avoidance of animal serum products during isolation, and a target islet mass of at least 11,000 IEQ/kg of recipient body weight, which requires islets from 2 to 3 donor preparations. They used an immunosuppressive protocol comprised of induction therapy with a humanized interleukin-2 (IL-2) receptor antibody (daclizumab) and maintenance therapy involving low dose tacrolimus and sirolimus.[29] More than 471 patients with T1D have received islet transplants at 43 institutions worldwide in the past 5 years.[14] High rates of insulin independence have been observed at 1 year in the leading islet transplant centers and an international multicenter trial has demonstrated reproducible success of this approach[14] (Fig. 1C). Nevertheless, provided the ability to correct hyperglycemia and maintaining the recipients insulin-free after one or more intraportal islet injections, the 5-year Edmonton protocol follow-up clearly indicates that the long-term function of the graft is lost or significantly reduced over time, with less than 10% of the patients remaining insulin-independent 5 years following transplantation.[31]

Prospective Problems of Islet Transplantation in the Setting of Autoimmune Diabetes

The success of islet allotransplantation in T1D patients is strongly subjected to the ability to appropriately control the occurrence of 2 pathophysiological events: allorejection and autoimmunity. The immunosuppressive medications used to prevent rejection by inducing a status of generalized suppression of the immune system can only partially and indirectly address the contribution of auto and alloimmunity and their combined effects. The possible mechanisms of destruction of the transplanted islets are therefore potentially numerous, occurring via a broader array of effectors. The role of the isolated islets themselves, their qualitative characteristics following isolation and transplantation, the impact of the implant site and of the procedure of transplantation represent additional causes of low islet graft survival in that they contribute to elicit a specific inflammatory responses that can, in turn, exacerbate both the auto and alloimmune response in the recipient. These immunologic as well as nonimmunologic factors need careful consideration.

IMMUNOLOGIC ISSUES IN ISLET TRANSPLANTATION

The Challenge of Recurrent Autoimmunity

In 1978, Connolly[32] noted that islet cells are more violently rejected in humans than other transplanted tissue. His statement was prophetic. Since then, it is now undisputable that the autoimmune background of a type 1 diabetic quite likely plays a decisive role in islet transplant rejection. Observations in human T1D recipients of islet allografts strengthened the argument that typical chronic allograft rejection processes could not singularly account for the rejection processes of islet allografts in T1 patients with diabetes.[22,33–38] This major impediment in islet allotransplantation success was additionally predicted in the Bio-Breeding (BB) and the nonobese diabetic (NOD) rodent models of the disease.[39,40] The exact cellular processes that distinguish the autoreactivity against the islet transplant from alloreactivity are not all that clear, but the available data seem to implicate class II Major Histocompatibilty Complex (MHC) in autoimmune processes.[41–43] At the molecular level, this would reflect either an activation of quiescent, long-lived memory-type autoreactive T-cells whose T-cell receptor (TCR) would be selected on one or more beta-cell-restricted epitopes presented on recipient antigen-presenting cell class II MHC, or the stochastic generation of beta-cell-reactive thymocytes following the transplantation procedure. Whichever mechanism is occurring, it is evident that the indirect pathway of antigen presentation by antigen-presenting cells of the transplant recipient plays the dominant role in the autoimmune rejection arm of transplant failure.

A number of strategies have been employed to prevent both arms of islet transplant rejection. Thus far, they have

involved the induction of allogeneic chimerism using hematopoietic cells, depletion of T-cell subsets, co-stimulation blockade, and pharmacologic immunosuppression targeting T-cells, with or without chimerism induction. In the BB rat model, a monoclonal antibody against $CD8^+$ T-cells (OX8) was successful in abrogating islet autoimmune rejection post-transplantation.[44] Antilymphocyte serum was shown to be effective at suppressing autoimmune rejection in NOD recipients of allogeneic islets, but it was less effective at allorejection.[45] Nondepleting anti-CD4 antibody treatment of NOD recipients of islet allografts demonstrated protective effects against autoimmune rejection; however, these beneficial effects were compromised in the presence of B7 co-stimulation blockade by CTLA-4Ig.[46] Similarly, depleting CD4 antibodies were effective in facilitating islet allograft survival in new-onset diabetes in the NOD mouse.[47] Molano and colleagues employed an anti-CD40 strategy to prolong allogeneic islet transplant survival in NOD mice with considerable success; however, in similar studies performed in BB rats, the effects of CD40 blockade on autoimmune rejection were not as effective.[48] Molano et al[49] have more recently shown that co-blockade of CD45RB and CD40 can be quite effective in abrogating autoimmune rejection processes in allogeneic islet transplantation.

The induction of mixed chimerism is believed to minimize autoimmune phenomena by augmenting the existing antigen-presenting cell population in a recipient with antigen-presenting cells that can compete for beta-cell-restricted antigens, or that can support the survival and generation of immune regulatory cells. Chimerism has resulted in suppression of autoimmune rejection of allogeneic islets in a number of instances, in processes that likely involve antigen-presenting cells and T-cell subsets.[50–55] The most unique method of abrogating autoimmunity involves the direct injection of allogeneic or syngeneic islets into the thymus of recipients.[56,57] Mechanistic understanding has not been extensively pursued, but it is possible that thymocytes destined to become central T regulatory cells ($CD4^+$ $CD25^+$), may encounter beta-cell-restricted antigens in a thymic micro-environment and thereby maintain a degree of robust suppression of autoreactivity in transplanted hosts—even in a background of beta-cell-specific autoreactive T-cells.

Finally, the Edmonton pharmacologic immunosuppressive protocol, described in the next section, exhibited considerable efficacy in the NOD mouse in prolonging islet allograft survival.[58] Mechanistically, much remains to be understood about how each component affects the cells likely responsible for autoimmune rejection, but based on the known molecular pathways affected by the constituents of the Edmonton immunosuppressive cocktail, there is a high likelihood of clonal exhaustion of autoreactive T-cells that are being arrested in the early phase of the cell cycle (and driven to apoptosis) in a rapamycin-dependent manner.

Pharmacological Antirejection Therapy in Islet Transplantation

The immunosuppressive regimen that has been used in islet cell transplantation has changed over the years; however, an optimal regimen has yet to be discovered. The first successful trial of human islet allotransplantation, as mentioned before, that resulted in the long-term reversal of diabetes was done at the University of Pittsburgh in 1990.[28] This initial study represented a trial of 9 patients who became diabetic after upper abdominal exenteration followed by liver transplantation and the infusion of allogeneic human islets. Early islet allograft function was seen in every recipient, which was sustained in 5 of these patients.[28] Some of the patients remained insulin-free for up to 6 years. The islets were transplanted using a steroid-free protocol and the new drug FK-506 (Prograf). The unprecedented success of this trial resulted in the resumption and initiation of additional clinical protocols for islet cell transplantation. Most of these transplants were done as solitary islet transplantation for T1D. The immunosuppressive regimen for most these cases consisted of antibody induction therapy with an antilymphocyte globulin combined with cyclosporine, azathioprine, and glucocorticoids.[29] Insulin independence, under such protocols, was minimal. Although the immunosuppressive regimen was suspected to cause a significant amount of islet destruction, there was insufficient knowledge about the effects of these drugs on islet survival and function. Most of the detrimental effects were blamed on steroid use.[59] Autograft studies done at that time demonstrated that prednisone had a detrimental effect on islet function but combinations of immunosuppression, which included azathioprine, antilymphocyte globulin, and cyclosporine, were not believed to be detrimental to islet function, resulting in hyperglycemia.[59] At this time, Prograf was just being introduced in Pittsburgh; therefore, little was known about its effect on islet function and survival. Due to these studies and others, leaders in the field believed that the use of steroids as part of maintenance immunosuppression or as treatment of acute rejection in islet transplantation should be reconsidered.[59] Due to these poor results, the enthusiasm for islet transplantation was significantly dampened. Then, in the year 2000, a significant breakthrough occurred. A new immunosuppressive regimen for islet cell transplantation, known as the Edmonton protocol, was launched. In the original Edmonton protocol, immunosuppression is initiated before transplantation.[29] Three drugs are used: daclizumab (Zenapax), sirolimus (Rapamune), and tacrolimus (Prograf). Sirolimus is given orally at a loading dose of 0.2 mg per kilogram per day, monitoring drug levels to maintain a trough level of 12–15 ng/mL for the first 3 months and then 7–10 ng/mL thereafter. Low dose tacrolimus is given orally at an initial dose of 1 mg twice daily to maintain a 12-hour trough level of 3–6 ng/mL. Daclizumab is given intravenously at a dose of 1 ng/kg every 14 days for a total of 5 doses (there is a current trend to continue this treatment every 2 weeks). For prophylaxis against *Pneumocystis carinii*, oral Bactrim is given 3 times a week. Valganciclovir is given daily (450 mg) for at least 12 weeks for prophylaxis against cytomegalovirus (CMV) (the length of treatment has been increasing recently due to the more potent antibody preparations). Patients also receive oral supplementation of vitamin E (800 IU/d), vitamin B6 (100 mg/d), vitamin A (30,000 IU/d), and vitamin C (1000 mg/d), for antioxidant

supplementation, which may protect the islets. Although this regimen has successfully resulted in the attainment of insulin independence, these agents have some significant side effects and have not resulted in reliable long-term survival of the islets. Daclizumab binds specifically to the IL-2 receptor that is expressed on the surface of activated lymphocytes. It is usually well tolerated but occasionally it is associated with some adverse effects. These may include mild gastrointestinal symptoms, dizziness, headache, tremor, changes in heart rate, and blood pressure, bleeding, musculoskeletal pain, and pulmonary edema.

Tacrolimus inhibits T-lymphocyte activation by binding to an intracellular protein, FKBP-12. A complex is then formed, which inhibits calcineurin. This effect is thought to prevent the translocation of nuclear factor of activated T-cells (NF-AT), which is involved in the initiation of gene transcription for the formation of lymphokines, such as interferon-2 and gamma interferon. It is fairly well tolerated at the low doses that are used in islet cell transplantation; however, it also acts synergistically with sirolimus. The precise pharmacokinetic effects on the islets are not entirely understood. Tacrolimus can cause neurotoxicity and nephrotoxicity, which are very much dose related. Hypertension can be fairly common but myocardial hypertrophy is rare. Hyperkalemia and hypomagnesemia are fairly common but easily treatable. One of the major concerns with tacrolimus is that it has been found to be diabetogenic. Because of these side effects, new protocols are being developed to try to avoid tacrolimus and other calcineurin inhibitors.

Sirolimus binds to the immunophilin FK binding protein to generate an immunosuppressive complex, which has no effect on calcineurin activity. This complex binds to and inhibits the activation of the mammalian target of rapamycin (mTOR), and this suppresses cytokine-driven T-cell proliferation. Some of the side effects associated with sirolimus include hyperlipidemia, hypercholesterolemia, diarrhea, hypertension, anemia, mouth ulcers, joint pain, impaired wound healing, thrombocytopenia, and proteinuria. A significant number of these patients require drug therapy for their elevated cholesterol and lipid levels. Mouth ulcers and poor wound healing are a significant problem, which sometimes requires dose reduction or a change in medication. Dosage reduction of tacrolimus has also been necessary in patients with rising creatinine levels and decreased creatinine clearance.

All immunosuppressive agents have the potential complications of infections, post-transplantation lymphoproliferative disorder (PTLD), and other malignancies (although sirolimus seems to be associated with a lower incidence of malignancy). Currently, in islet cell transplantation, there have been no cases of Epstein–Barr virus (EBV), CMV, post-transplant lymphoproliferative disease (PTLD), or cancers (related to immunosuppression).

Most programs still follow the original Edmonton protocol; however, newer protocols are currently being developed due to the disappointing long-term results and the significant side effects associated with these agents. These new protocols include antibody induction, co-stimulatory blockade, and calcineurin-inhibitor avoidance, in the hope of reducing these side effects and increasing graft survival. Some of the antibody preparations that are being introduced into these protocols include thymoglobulin, OKT3, and Campath-1H. There is a great deal of enthusiasm for agents that interfere with cellular signaling for lymphocyte activation, deplete T-cells, and alter lymphocyte trafficking and recruitment. Medications such as FTY 720, Everolimus, and Cellcept are being used in numerous combinations in these regimens. The current trend is to develop immunosuppressive protocols that are calcineurin-inhibitor-free.

Besides the changes in the immunosuppressive regimen, additional medications that are biologic or immunomodulatory are also being investigated. Inflixumab, which is commonly used for the treatment of Crohn disease, neutralizes the biologic activity of tumor necrosis factor (TNF) alpha. This drug is given before islet cell transplantation to decrease inflammation to achieve an increased survival of the islets. In the same manner, another drug, Etanercept, is also being studied. This drug has been used in psoriasis and rheumatoid arthritis patients. It binds to TNF alpha and blocks its interaction with cell surface TNF receptors. These drugs are primarily being used in islet transplantation as anti-inflammatory agents; however, they do have some immunomodulatory-immunosuppressive effects. The rationale for using these agents is that there may be a significant amount of islet destruction due to inflammatory events, which are occurring during organ recovery, isolation, and transplantation. Reducing this inflammation may result in an increased graft survival using fewer islets.

In addition to these immunologic and inflammatory events, there may be other reactions that can destroy the islets, which need to be addressed in these protocols. A unique thrombotic-inflammatory reaction has been described recently, which is thought to be elicited when the islets come in contact with ABO-compatible blood.[60] This reaction, described as an immediate blood-mediated inflammatory reaction (IBMIR), may provide an explanation for early islet loss. To what extent the islets are lost in this reaction versus purely immunologic phenomenon is not known at this time. In addition, this phenomenon is currently controversial. Some investigators deny its existence whereas others believe that it is a critical reaction that must be treated. A confounding variable in the debate is that all centers use heparin, which is an antithrombotic agent. It may be possible that heparin mitigates this reaction and therefore it may not always be appreciated. Because of this presumed reaction, several anticoagulant agents are being studied to see if they can reduce or ameliorate this effect.

Because of these developments and the continued work on the effects of inflammation in general, the newer protocols for islet cell transplantation may contain these anticoagulant and anti-inflammatory agents, along with some newer immunosuppressive agents. It is very possible that these anticoagulant and anti-inflammatory medications may be just as necessary as the usual immunosuppressive drugs. There may be a complex interaction with overlapping reactions between the coagulation and immunologic systems, which involves complement, cytokines, and numerous inflammatory mediators. Therefore, to achieve

the goal of prolonged graft survival, new protocols will most likely require specific combinations of anti-inflammatory, anticoagulant, and immunosuppressive drugs. It may be possible after further investigation that in islet cell transplantation, the anti-coagulant and anti-inflammatory agents may play an even greater role than the standard immunosuppressive drugs because we know that some of these agents have been detrimental and even diabetogenic. The challenge will be to find the best protocol with the least toxicity while continuing to improve our isolation and transplantation techniques.

NON-IMMUNOLOGIC PROBLEMS AFFECTING ISLET PERFORMANCE

Pancreas Procurement and Preservation

The first stage of islet transplantation is the procurement of a high-quality donor pancreas. Successful isolation of pancreatic islets depends on careful procurement of the organ. Typically, the pancreas is procured from a cadaveric heart-beating brain dead donor. The process of brain death can itself prove detrimental to the human pancreas.[61] Islet recovery, purification, and functionality of the isolated cells largely depend on the organ procurement method. Combined removal of the liver and pancreas is the standard procurement procedure currently used.[62–64] The pancreas is separated from the block in a second step. Minimal cold ischemia time (<12 hours), following pancreas procurement, is vital for islet survival after isolation whereas whole pancreatic grafts prove functional even after 24 hours of cold storage. University of Wisconsin (UW) solution, Eurocollins, and histidine–tryptophan–ketoglutarate (HTK) are the major solutions used for pancreas preservation. The Edmonton group reported a distinctive method of pancreas retrieval to better suit the qualitative requirements for successful islet isolation and transplantation. They correlated the pancreas procurement technique with variations of the pancreas temperature and islet functionality.[65] They concluded that maintaining a low pancreas temperature during procurement through the addition and replenishment of iced saline slush surrounding the anterior and posterior areas of the pancreas greatly improves islet yield and functional viability of the isolated islets and is essential for success in clinical islet transplantation.[65] Another important aspect to consider during pancreas harvest is avoiding distress handling of the organ and retaining the pancreatic capsule intact. A damaged pancreatic capsule will not hold the infused isolation enzyme during ductal injection. Inflation with enzyme and adequate distension of the pancreas is very crucial for proper islet release.[66]

Currently, a 2-layer method using perfluorocarbons (PFC) and UW solution is common practice to reduce cell damage by increasing oxygen supply during cold storage preservation of the pancreas.[67,68] Kuroda et al[69] first introduced the 2-layer cold storage (TLM) method for the preservation of the pancreas in animal models. Matsumoto et al[70] and Hering et al[71,72] introduced the PFC-based preservation method before clinical whole pancreas and islet transplantation. The efficacy of this method was tested on stringent conditions such as marginal human pancreatic organs,[73] including older pancreas donors, non-heart-beating donors,[74] or after prolonged periods of cold storage.[75]

The Impact of the Isolation Procedure

Islet isolation is a time consuming procedure required to purify the islet cells from the exocrine compartment of the pancreatic gland. The extraction procedure, now regulated by the Food and Drug Administration (FDA), involves a digestion as well as a purification phase. The islets are separated from the exocrine tissue by the chemical activity of collagenases and neutral proteases that are infused in the pancreatic duct in solution, allowed to reach the temperature of 37°C, and become chemically active. Exogenous enzymes mediate the cleavage of the extracellular matrix proteins of the pancreas that surround the islets. The enzymatic digestion of the pancreas is usually carried out in an *ad hoc* digestion device that contains the pancreas, maintains the recirculation of the enzyme solution, whereas mechanical shaking ensures a gentle disruption of the tissue.[76]

Once the islets are freed from the surrounding exocrine, they are separated from the exocrine tissue by means of a purification step based on the density difference between acinar and islet cells. A successful islet cell isolation technique was introduced by Lacy and Kostianovsky[77] in the year 1967. Rat islets were isolated by distending the pancreas with physiological salt solution, chopping the pancreas into small fragments and mechanical agitation of pancreatic tissue with the enzyme collagenase. Later, this method was successfully applied to various higher mammals including dogs,[78] pigs,[79] and monkeys.[80] Grey et al[81] reported an effective method of islet isolation from the human pancreas. In 1989, Ricordi et al[27] invented an automated digestion device for islet isolation that allows for reproducible high islet yields from the human pancreas. The initial step of human islet isolation is the digestion of the pancreas with the use of collagenase enzyme. This is achieved by intraductal injection and distension of the pancreas with enzymes. The infused enzyme solution flows through the pancreatic duct and reaches the extra cellular matrix regions spreading throughout the pancreatic tissue. The next step is a digestion phase characterized by enzymatic activity and mechanical shaking finalized to break the tissue; such procedure is carried out in a digestion device: the Ricordi's chamber. The digestion chamber is inserted in a closed circuit where the enzyme solution, warmed to body temperature, is allowed to circulate through the areas that contain the pancreas.[27] The circuit can be opened when the islets, now freed from the surrounding exocrine tissue, require to be collected in cold, serum-supplemented medium, to discontinue exposure to digestive enzymes.

The most prevalent isolation enzyme is collagenase, derived from bacterial cultures of *Clostridium histolyticum*,[82] a mixture of several different proteolytic enzymes. The heterogeneity of collagenase preparations and the immense variability between human donor pancreata continue to hamper a process that is inherently difficult to control.[83] An important advance has been the use of purified enzyme blend Liberase-HI that has low levels of endotoxin content and provides

consistently better islet yields. Several studies have shown that endotoxins have a detrimental effect upon islet cell engraftment[84] and activates proinflammatory cytokine gene expression.[85] Before the introduction of Liberase-HI, isolation reagents with high endotoxin content were common and most likely contributed to primary nonfunction of islets after transplantation.[86] Endogenous protease activity of the donor pancreas during collagenase digestion also affects the final islet yield and function. Lakey et al[87] successfully used Pefabloc, a serine–protease inhibitor, for pig and human islet isolation to reduce the activity of endogenous pancreas proteases. Currently, a purified form of good manufacturing practice (GMP) grade collagenase and neutral protease is used for human islet isolation, an alternative to Liberase-HI.[88,89]

Because islets are lighter than acinar cells, when the principle of density gradients is used, the islets are separated from the acinar. Human islet purification is efficiently achieved by using the COBE 2991 cell separator that was first introduced for human islet separation by Lake et al.[90] In the COBE machine, the pancreatic digest is centrifuged with density gradients (continuous or discontinuous) so to separate islet from exocrine-enriched fractions. Purified islets are favored as they reduce the cell mass infused and consequently the graft immunogenicity.[91] The commonly used gradients are Ficoll,[92] bovine serum albumin,[93] dextran,[94] hypaque-Ficoll,[95] Percoll,[96] sodium diatrizoate,[97] and metrizamide.[98] After purification, islets can be transplanted immediately or cultured for a short period before implantation. A culture period provides sufficient time to perform sterility and in vitro functional assessment of islets.

The isolation procedure itself causes loss of islet mass as a direct effect of the destructive activity of the enzymes and the inability to efficiently separate islets from the exocrine tissue without islet loss.[99] Moreover, conditions that characterize the donor, such as age, cause of death, long ischemia, and medical status, are equally responsible for the quality of the islets. Studies indicate that simple breakage of the extracellular matrix has a negative impact on islet survival.[100] Recently, additional factors that carry a potentially deleterious impact on the islets have pointed to the isolation procedure. Despite efforts to optimize the conditions of pancreas preservation *ex vivo*[71,73,101] and the islet isolation process as a means to improve islet yield, only a significantly limited part of the islet pancreatic content survives the process of isolation and subsequent culture. Although the cascade of events occurring during isolation of pancreatic cells, which may cause beta-cell dysfunction and ultimately death, is not fully characterized, recent lines of research have indicated in rodents[102,103] as well as in humans islets[104] that oxidative stress plays a major role in triggering death of the islets and of the surrounding exocrine tissue. Other reports have demonstrated that oxidative stress is strongly connected to the adverse effects of chronic hyperglycemia on insulin biosynthesis by islet beta-cells.[105]

It has been widely reported that islet beta-cells are highly susceptible to oxidative stress because of their reduced levels of endogenous antioxidants.[106] Under extreme conditions of stress, the islet antioxidant defenses may become overwhelmed, leading to a state of redox imbalance and production of reactive oxygen species (ROS). One potential ROS-dependent target molecule is the nuclear transcriptional factor NF-κB. It is now known that NF-κB is a key transcription factor involved in regulating proinflammatory cytokines, chemokines, adhesion molecules, and inflammatory enzymes. Blockage of NF-κB, by administration of an NF-κB decoy or by using antisense oligonucleotide treatment, protects beta-cells from the effect of IL 1-β -induced $NO^{\cdot}$ production.[107,108] Furthermore, it has been demonstrated that the native enzyme manganese superoxide dismutase (MnSOD) delivered to mouse islets by gene therapy approaches proved beneficial in improving islet cell survival after transplantation.[109]

It is our observation that in human islets the isolation process triggers the activation of NF-κB and PARP pathways that lead to apoptosis of the beta-cells.[110] As a consequence, cytokines and chemokines such as MCP-1 (macrophage chemoattractant protein-1) and IL-6 are expressed and released by the islet cells.[110] This phenomenon predisposes the islets to be target of intense a specific inflammatory responses following transplantation, even prior, and in addition to the allospecific cell-mediated immune response. The good news is that addition of potent antioxidants during the isolation procedure exerts a protective role on isolation damage to islets and warrants higher survival rates[104] when used as culture supplements[104,110] (Figs. 2 and 3). Besides islet cell loss, the effect of isolation stress seems to be relevant to islets once transplanted in the recipient.

Further observations shed light on the negative effects that enzyme digestion exhibit on the quality and function of the islets beyond the isolation phase. We have observed that Liberase-HI (the most used enzyme blends specifically formulated for human islet release) and its by-products retain the ability to penetrate and dwell in the cytoplasm of beta and other pancreatic cells after intraductal enzyme delivery[111] (Fig. 4). Exposure of isolated human islets to Liberase-HI, at

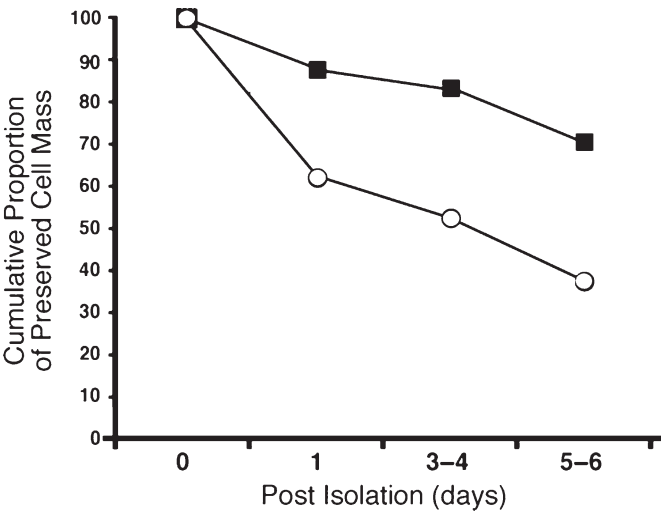

FIGURE 2. Addition of antioxidative compounds (SOD-mimic) during human islet isolation reduces the islet cell loss. Graph illustrates islet survival after antioxidant treatment (SOD- mimic) (close square) and control conditions (open circle). Reprinted from Bottino et al,[104] with the permission of the American Diabetes Association.

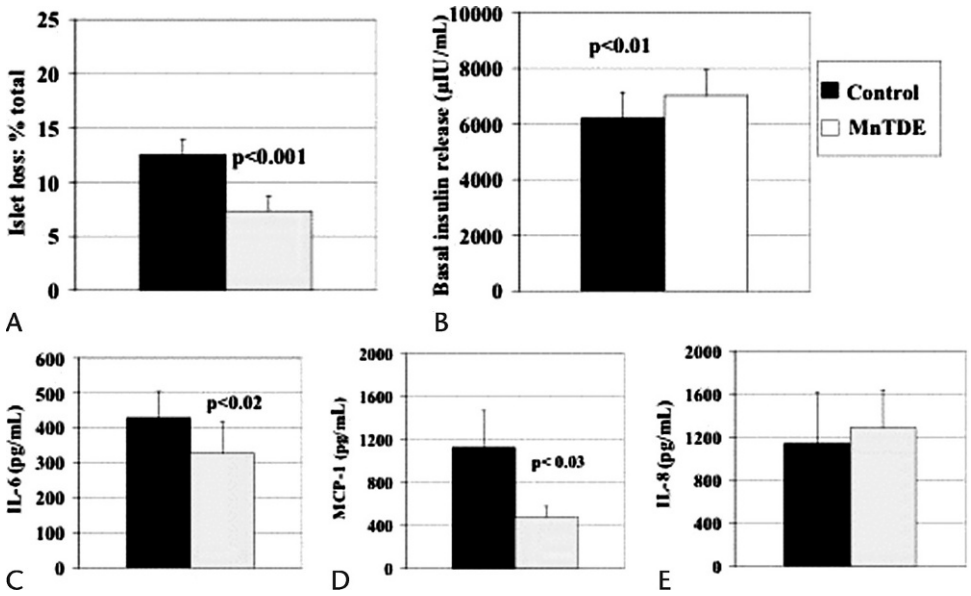

FIGURE 3. Presence of antioxidative SOD-mimic (MnTDE) in the culture milieu of human islets decreases the release of proinflammatory cytokines and chemokines. It is also increases the islet basal insulin secretion and prevents the islet cell loss in culture (MnTDE-treated group in open bar and control in close bar). Reprinted from Bottino et al,[110] with the permission of the American Diabetes Association.

the enzyme concentrations used during the isolation process, causes a reduced ability to release insulin in response to glucose, a deficit that correlates with the duration of exposure.[111] We have also seen that exogenous enzymes trigger the activation of proapoptotic proteins and the expression of adhesion molecules (CD106, CD62p) in islet cells during culture. In transplantation studies using NOD*scid* mice, chemically rendered diabetic and used as recipients of human islets, prolonged exposure of the islets to Liberase-HI caused more intense inflammation at the site of transplantation and was associated with significantly higher sickness behavior and even death of the recipients.[111] New formulations of exogenous enzymes for the isolation of human islets for clinical use have been recently tested showing improvements in islet morphology, viability, and in vitro function, compared to classic enzyme blends.[89]

Transplantation Site

Since the first successful isolation of pancreatic islets accomplished by Lacy and Kostianovsky[77] in 1967, many recipient sites have been proposed for islet transplantation. The optimal site for transplantation of islets has not yet been defined, although there is general agreement that the implant site should provide the adequate microenvironment, vascularization, and nutritional support to maximize the chances for a good islet cell engraftment and to minimize morbidity. Several transplantation sites for islet engraftment have been reported in various experimental animal models, including peritoneum, blood vessels, intrathecal areas, pancreas, salivary gland, brain, muscle, spleen, liver via the portal vein, mammary fat pad, anterior eye chamber, omental pouch, testis, and renal capsule.[112] Commonly, these sites can be classified into 2 groups: sites with systemic venous drainage and sites with portal venous drainage. In smaller animal models, the renal subcapsular site has been widely used but is unlikely to be adopted clinically and this environment is suboptimal with regard to islet cell oxygen tension.[113] The liver, reached via intraportal islet infusion, has been the most commonly used site for clinical islet transplantation because of the early success with autologous islet transplants. The intraportal infusion of islets is the only technique that has successfully led to insulin independence following islet transplantation in humans. The Islet Transplant Registry reported that 93% of islet allotransplantations in T1D patients have been delivered via the portal vein site.[25]

The islets are implanted into the portal system of the liver using minimally invasive interventional radiology techniques. Percutaneous hepatic cannulation is the standard approach.[114] Under local anesthesia, the patient's portal vein is located through radiologic and ultrasound guidance and a catheter is passed into the main portal vein using a guide wire.[114] Islets are infused although the tube by gravity flow from an infusion bag[115] or a syringe.[116] While the islet suspension is slowly infused, the portal pressure is monitored periodically. Transient portal hypertension may occur because of embolization of islets in the liver. Thrombostatic coils and hemostatic agents are used to plug the catheter opening. The risk of significant hemorrhage and portal vein thrombosis after percutaneous islet transplantation, although rare, are the major concerns. Rise in liver function tests (especially aspartate aminotransferase and alkaline phosphatase) and puncture of the gallbladder are also possible events associated with the transplant procedure.[114]

In the case of simultaneous islet and kidney transplantation, islets are directly injected into the portal system during an

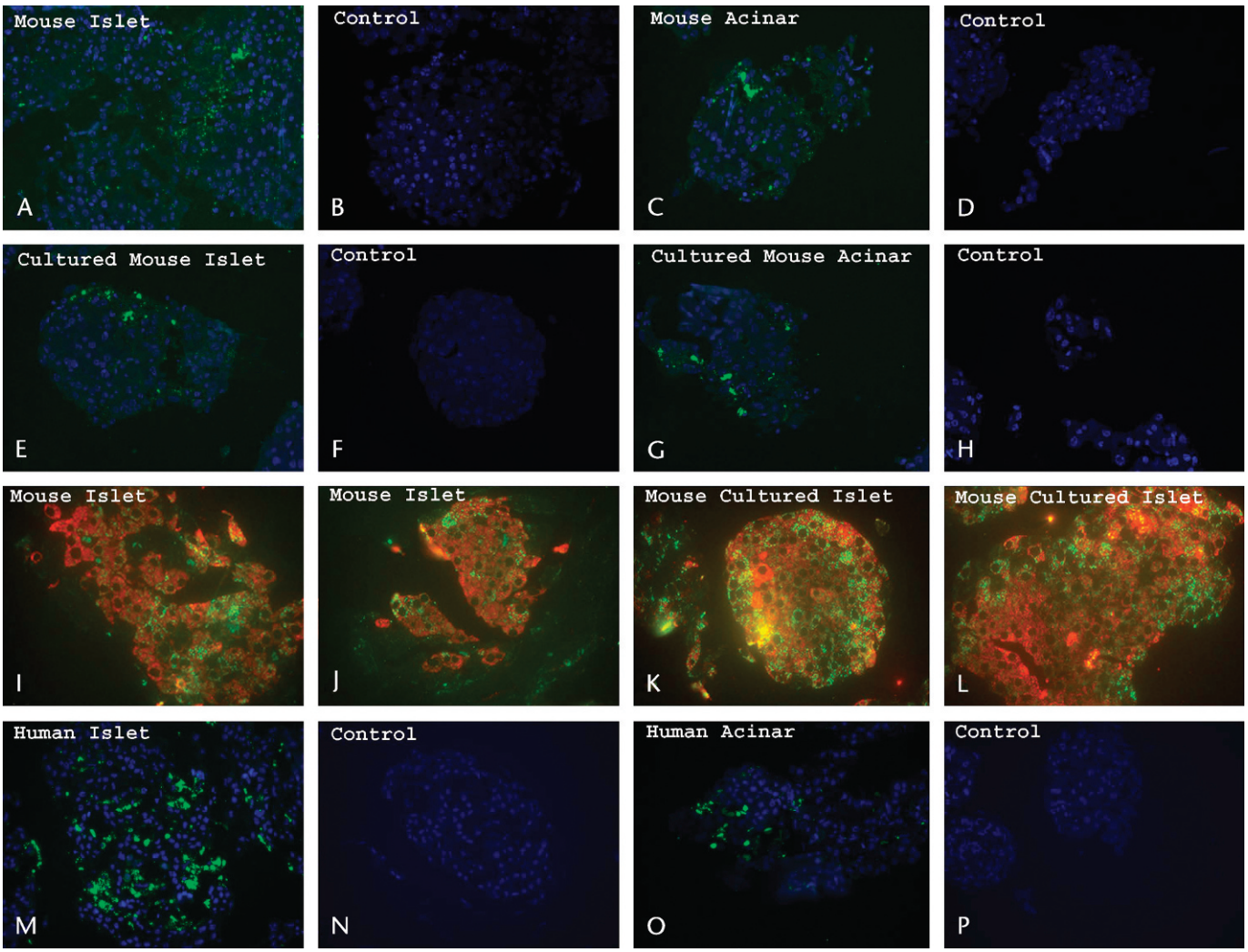

FIGURE 4. Intracellular traces of fluorescein-conjugated Liberase-HI after isolation and culture of mouse and human pancreatic islet and acinar cells. (Green color indicates fluorescein-conjugated Liberase. Insulin is shown in red.) Reprinted from Balamurugan et al,[111] with the permission of the Blackwell Publisher.

open surgical procedure. Although the intraportal site has several advantages, a significant reduction in the mass of transplanted islets occurs immediately after transplantation in the liver of recipients. A major cause of islet cell loss is, as mentioned before, the instant IBMIR characterized by rapid platelet deposition on the islet surface, activation of the coagulation and complement cascades, and leukocyte infiltration of the islets.[117] Islet cells produce tissue factor, which is a triggering mediator of the coagulation cascade.[60] In the hepatic microenvironment, islets are constantly exposed to hypoxia and consequently to local inflammation through the activation of endothelial as well as Kupffer cells. These promote generation of proinflammatory cytokines, oxidative stress mediators, and recruitment of leukocytes, all of which may contribute to enhancing islet injury early after islet implant.[118] Kupffer cells and the beta-cells themselves secrete many molecules including cytokines, NO, and free radicals, which are known to be directly toxic to the pancreatic islets.[119] Furthermore, intrahepatic islets may be exposed to environmental toxins and immunosuppressive drugs absorbed from the gastrointestinal tract and delivered into the portal vein. Perturbation of the liver cells, consequent to islet engraftment, has also been observed and includes hypertrophy of the hepatocytes and fibrosis.[120,121] Potential alternative sites include the peritoneal cavity and omentum, both of which have been used successfully in animal models and shown to be safe for humans.[30]

RECENT DEVELOPMENTS AND CHALLENGES

Optimization of the immunosuppressive protocols, appropriate selection of donor–recipient size and improved culture conditions for the islets before transplantation, is leading to a steady improvement of the islet transplantation outcome, allowing normalization of glycemia in diabetic patients even after infusion of islets from single living or deceased donor, and allowing islet transplantation between remote centers.[122,123]

Broader application of islet allotransplantation, however, will not be achieved without appropriate solution to 2 major problems:

i. the need for alternative, minimally toxic immunosuppressive protocols; and
ii. the release from the autoimmune pressure.

An increased applicability of this procedure would immediately raise the problem of donor shortage and availability of islet cell sources. Concerning the organ donation programs, it is already clear that the disparity between organ supply and demand will remain unsolvable. In the United States, the number of newly diagnosed cases of T1D annually is 30,000 and the number of donor pancreata is approximately 6000.

The possibility to explore islet animal sources or establish protocols for the proliferation and differentiation of insulin producing cells from non-pancreatic sources from autologous or heterologous donors is already object of research studies,[3] as well as the selection of transplantation sites where islets could find a more appropriate physiological environment and that could be eventually retrieved at later stages.

The insufficient availability of human donors for islet transplantation is therefore one of the compelling reasons to exploring the ability to generate insulin-producing cells from a variety of cell sources. Fascinating but yet unsuccessful approaches involve attempts at ex vivo expansion of beta-cells or controlled differentiation of animal and human embryonic stem cells into insulin producing cells. One of the major obstacles to achieve efficient generation of beta-cells in vitro is the difficulty in identifying the natural beta-cell precursors in the pancreas. A number of cell types, undifferentiated cell subpopulations, as well as fully differentiated cells of the pancreas (either the beta-cell itself or alternately ductal and acinar cells with the potential to transdifferentiate) have been proposed to contribute to the formation of beta-cells; however, the current knowledge is still controversial. Transplantation studies would strongly benefit from the identification and isolation of progenitor cells from the adult pancreas, in particular, if these cells could be isolated also from the pancreas of the patient himself, with the prospective to implant autologous, ex vivo expanded, beta-cells.[3,124–126]

A new regenerative hypothesis envisions that the endogenous pancreas maintains the ability to resupply, at a very low pace, new insulin producing cells to compensate for the beta-cell mass lost as a consequence of autoimmune or other toxic injury.[3] The favorable conditions and the extent of this phenomenon are still mostly unknown and require careful investigation. Whether the potential of self-healing process proofs critical to be exploited as a possible curative, novel therapy for T1D, it is not clear but offers very exciting prospectives. In this contest, a role of islet transplantation, either as provider of possible trophic factors or to relief the diabetic individual from insulin demand during the phase of self-regeneration of the insulin producing cells, as shown in animal experiments,[127] could still be critically important.

ACKNOWLEDGMENTS

The authors are grateful to Amy Sands and Carmella Knoll for helping with the manuscript preparation.

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