Gene therapy technology applied to disorders of glucose metabolism: promise, achievements, and prospects

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Gene transfer technology has spawned an entire realm of clinical investigation, collectively referred to as "gene therapy." The feasibility and achievements of gene therapy to prevent and treat glucose homeostasis disorders, with particular emphasis on diabetes mellitus, are evaluated in this review. While a considerable amount of effort has yielded gene delivery vectors based on adenoviral, retroviral, and herpes simplex virus DNA, the number of successful clinical applications has not been as impressive. Despite the number of successes in vitro and in animal models, preliminary safety trials in humans have not yet been attempted. The current state of this science, outlined here, underlines the necessity of marrying gene transfer technology with cell therapy. The ex vivo transfer of gene combinations into a variety of cell types will likely prove more therapeutically feasible than direct in vivo vector transfer. Current efforts aimed at assessing the future of gene therapy for diabetes must, at the very least, take into account the importance of moving successful methods into human safety trials.

INTRODUCTION

Diabetes mellitus refers to a multi-type disorder of glucose homeostasis defined by the loss of insulin production and/or the insensitivity of peripheral tissues to the effects of insulin. Generally, the disorder falls into two categories: type 1 and type 2 (1–4). The loss of insulin due to the autoimmune destruction of the β cells defines type 1 diabetes mellitus. Impaired glucose sensitivity and/or insulin production by pancreatic β cells in the presence or absence of poor sensitivity to the effects of insulin in the periphery define type 2 diabetes, which is commonly diagnosed in adults. Often, obesity is a significant risk factor for type 2 diabetes (5–7). Today, the conventional treatment for type 1 diabetes remains insulin replacement. Pharmacologic agents acting at the level of the β cell to improve glucose sensitivity and insulin production, along with drugs that can sensitize the peripheral tissues to the effects of insulin, are the forefront of the

effort aimed at restoring good glucose control in type 2 diabetics. In addition, several bioactive peptides have also been discovered that have potent effects on insulin production and are currently in various stages of clinical development (8–10). However, insulin replacement and the various pharmacologic agents cannot be considered "cures." Despite the significant improvements in glycemic control, these strategies are unable to achieve permanent, normal, physiological glucose control. The consequences include diabetes complication-associated morbidity and mortality (11). In fact, nonphysiologic glucose control is a predominant risk factor for cardiovascular, renal, and ophthalmic diseases. Where traditional pharmacological therapies fall short, bioengineering strategies offer an alternative.

Gene transfer technology is the cornerstone of gene therapy, widely cited as an alternative approach to, or as a complement of, traditional pharmacotherapy in several human disorders.

Indeed, a number of studies in animals have demonstrated the potential of this technology in type 1 diabetes (12,13). While advances in metabolic engineering of cells have not kept pace with those in the field of immune regulation; nonetheless, several approaches for engineering surrogate β cells are equally promising. In this review, we outline the technologies that have been used in gene therapy strategies for disorders of glucose homeostasis, with a special emphasis on diabetes mellitus. We illustrate the successful applications of gene transfer technology and current limitations. We conclude with some proposals that could serve as the basis of further investigation.

DISORDERS OF GLUCOSE HOMEOSTASIS

While several human disorders have impairments in glucose homeostasis (hyperglycemia/hypoglycemia) as their symptoms; often, this is a consequence

of the disorder and transient. Primary chronic hyperglycemia is synonymous with type 1 and type 2 diabetes mellitus, and type 2 diabetes is closely associated with obesity. To uncover targets of intervention, it is important to understand the molecular mechanisms of the etiology and progression of these disorders.

Type 1 diabetes is an autoimmune disorder whose onset occurs at a very early age and, in most patients, results in pancreatic β cell impairment or destruction by the time of diagnosis, which requires insulin replacement. It is a T cell-driven disease that relies on the presence of specific HLA alleles that are often found clustered in families (1,2,14,15). Despite the confirmation that the disorder has a genetic basis, the significant concordance between twins and the mapping of susceptibility loci, type 1 diabetes is thought to involve an environmental trigger (2,15). The T cell specificity for pancreatic β cell antigens exposes an underlying defect of central tolerance mechanisms and, very likely, dysfunctional peripheral immunoregulation.

In contrast, type 2 diabetes is consequence of several, often overlapping, physiological and cellular defects, with only sporadic evidence of an autoimmune component (16). Type 2 diabetes mellitus is associated with insulin deficiency and insulin resistance. It is still unclear where the primary defect lies and which of the two is a secondary response, although many studies support insulin deficiency as secondary to peripheral resistance (3,17). Genetic and environmental factors contribute to the etiology of type 2 diabetes mellitus, with the concordance rate in identical twins at 90% with variable prevalence among different populations. More than 50 genes have been identified that are involved in glucose homeostasis, which suggests that the cause of type 2 diabetes is heterogeneous and will likely involve defects in many pathways that play a role in insulin expression, secretion, and sensitivity and that even this alone will define only a subset of cases (3,17).

Obesity is probably the most important environmental risk factor for type 2 diabetes. Obesity induces insulin resistance in both humans and animal models of type 2 diabetes (5,18,19). Several

genetic defects have been discovered that implicate the leptin pathway as a key regulator of satiety and obesity (18,19). Leptin-deficient and leptin receptor-deficient mice and rats are obese and exhibit many characteristics of type 2 diabetes to varying degrees. The importance of leptin in human obesity, insulin resistance, and type 2 diabetes, however, remains to be determined, although there is no current evidence to support leptin defects as causal of human obesity or diabetes (18,19).

A role for free fatty acids in insulin resistance and type 2 diabetes etiology has been proposed by a number of studies, following the observation that chronic elevation of serum-free fatty acid concentrations in obese or diabetic individuals is associated with decreased glucose uptake into peripheral tissues (20–22).

GENE THERAPY TECHNOLOGY

It is evident from the physiology that some tissues and cell types are targetable by, and exploitable for, gene engineering to achieve one or more desired therapeutic benefits. For type 1 diabetes, the cells of the immune system can be targets of molecular vehicles that carry genes or proteins able to induce immunosuppression in a spatially confined manner and with restricted tissue distribution. In parallel, transplantation of insulin-producing tissues can be engineered to resist rejection with no need for the chronic administration of toxic immunosuppressive drugs. Additionally, understanding the process of T cell maturation can assist in designing gene engineering strategies that can prevent the onset of autoimmunity.

While it may not be readily apparent why gene therapy can or should be applied to type 2 diabetes, most of the pharmacologic interventions used to treat insulin resistance (a preface to overt diabetes) eventually fail, which suggests that a targeted approach may be more able to prolong the time to onset of insulin resistance or prevent it altogether. While “insulin resistance” genes have yet to be identified, type 2 diabetes has been associated in rodents and humans with specific mutations/

polymorphisms of glucoregulatory, glucose-sensing genes, or is preceded by obesity and insulin resistance that may or may not be underlined by genetic susceptibility (3,23), which suggests that gene therapeutic strategies aimed at type 2 diabetes should be based on a knowledge of the biochemistry of glucose-sensing, insulin sensitivity, and fuel metabolism. Targeting one or more of these pathways may achieve the identical results as current pharmacologic therapy, with no risk of the toxicity inherent in some of the more aggressive and potent agents currently in clinical use.

To deliver genes into cells and tissues, it is necessary to identify the “engineerability” of a cell; whether it is a dividing cell, differentiating cell, cell with limited lifespan, or cell with high metabolic activity. These factors will determine the choice of vector system that will facilitate the conferring of an exogenously demanded activity to a cell. Currently, vector systems fall into two broad categories, each with their advantages and limitations. We will describe these in the context of gene therapy for diabetes mellitus. Table 1 illustrates the general properties of the two vector categories, viral and nonviral.

Viral Vectors

The generation of viral gene delivery vectors requires two major components: vectors encoding a complete, or components of, viral genome sequence that is engineered in such a manner that no replication-competent particles can be generated. Additionally, the gene or genes that achieve the desired therapeutic or prophylactic objective are supplied as part of the viral sequence or separately in another DNA sequence. The second component is a cell line that can efficiently produce recombinant virions that do not have the capacity to replicate once they infect a host. Several strategies are currently employed to generate replication-defective adenoviral vectors (Figure 1) (24–34), and some are commercially available. Table 1 outlines the reasons adenoviruses are versatile. Their most important feature is the ability to generate these vectors in high titers and to infect a broad range of cell types (24,25). It is therefore not

surprising that the greatest number of gene therapy approaches in vitro and in vivo have been carried out with adenoviruses. The major limitation of these vectors is their immunogenicity, which is very likely dependent on the presence of expressed sequences in the vector backbone (24,25). If the aim is to generate a state of localized immunosuppression, then such a feature is intuitively counterproductive. Several strategies have succeeded in generating “gutless” adenoviral vectors, but to date, it has been challenging to grow these vectors to high titer without the presence of contaminating helper virus (24,25). Adeno-associated vectors offer advantages over adenoviruses, and recent data suggest their potential benefits (35–40). What is clear is that the utility and versatility of novel viral vectors are ultimately compared to that of adenoviruses; generally, adenoviruses are the benchmark.

Retroviral vectors were among the earliest choice vectors in gene therapy models. They easily infect replicating cells and integrate into the host genome. Actively replicating immune cells, especially T cells, were among the first cell types to be transduced by retroviruses. Their generation is straightforward, and several cell lines and processes are currently commercially available. Primarily based upon the Moloney murine leukemia virus (MMLV) genome, retroviral vectors have evolved into embodiments of avian, bovine, feline, equine, and human retroviruses (27–30,32,34).

The properties of human immunodeficiency virus (HIV)-1 were exploited to develop the first vectors of the *Lentiviridae* family. Lentiviral vectors

Figure 1. Construction of viral vectors. (A) Adenoviral vectors. Cell lines expressing adenoviral helper and packaging functions are transfected with transfer plasmids encoding the transgene of interest. Recombination of homologous sequences occurs within the cells. Newer developments take advantage of recombination within appropriate bacterial hosts, minimizing nonrecombinant vector production in cells. (B) Retroviral vectors. Transfer plasmids encoding transgenes are transiently transfected into packaging cell lines. (C) Herpes viral vectors. To construct a vector that is ICP4⁻ and ICP22⁻ (deletions in crucial genes that are necessary for replication competency), ICP4⁻ viral DNA is transfected into a producer cell line with a transfer plasmid in which the transgene cassette is cloned inside the deleted ICP22 locus.

offer low to no immunogenicity, but their cell range is limited, and the titers achievable on a packaging cell basis are significantly lower compared to adenoviruses. In the diabetes context, they can infect cells that are poorly transducible by adenoviral vectors and offer the advantage of stable genomic integration. This is a desired feature in approaches aimed at engineering cells that do not divide, differentiate, and are long-lived (27–30,32,34).

A limitation of all the aforementioned vectors is the transgene size that can be accommodated inside the vector backbone. Where strategies envisage multicistronic transgenes and/or promoters of complex (and long length) structure, solutions are provided by the herpes simplex viruses (26,28,41). While earlier generations of these vectors were toxic to transduced cells, current embodiments do not promote significant levels of cell toxicity in vitro or in vivo (42–46). A disadvantage is the cumbersome process required to generate significant titers, but ongoing efforts at large-scale vector production promise to overcome this limitation (42–46).

Nonviral Vectors

While viral vectors are versatile for almost all gene therapy applications, the immunogenicity, toxicity, potential for replication competency, the potential of wild-type helper virus carryover during production, and the sheer logistics required to maintain good manufacturing procedures/good laboratory procedures facilities for clinical-grade vectors are considered to offer more challenges than solutions. Nonviral vectors offer an inexpensive alternative. Traditionally, the transfer of naked DNA plasmids was synonymous with nonviral vectors; however, several recent developments have welcomed new types of gene transfer vehicles that contain no viral elements.

Naked recombinant DNA plasmid generation is rapidly scalable with considerably lower cost compared to viral vectors. Newer generation plasmid vectors have been designed to either stimulate immune responses or to have a low immunogenic profile (33,47–51). Additionally, multicistronic vectors are easily achievable compared to chal-

lenges to generate the same in viral vectors. Nonetheless, naked DNA cannot attain stable transgene persistence in transduced cells, and high concentrations are often required in multiple dosings (Table 1).

Newer approaches have succeeded in exploiting amino acid sequences that act as a cell entry facilitator of intact proteins. These peptide transduction domains (PTDs) are derived from viral nuclear import proteins and, unlike viral and plasmid DNA vectors that require gene transcription and translation for transgene expression, the PTDs are fused to the protein of interest. PTD-fused proteins can be readily generated in high yield with relatively minimal logistical requirements (52–56). The nature of the PTD domain can be modified to improve cell-type targeting. Their limitation, however, lies in their relatively short half life, as they are subject to proteasome-dependent proteolysis. Whatever the choice of vector, all have been tested in the context of gene therapy for diabetes, with varying outcomes.

THE PROMISE, ACHIEVEMENTS, AND PROSPECTS

The most obvious form of physiological glucose regulation in diabetic individuals has been insulin replacement. It is now evident that pharmacologic insulin replacement, although able to achieve tight glucoregulation, cannot prevent the complications. The alternative that many have aspired to has been to facilitate islet transplantation or to engineer surrogate β cells. While the recent successes achieved by glucocorticoid-free immunosuppression have propelled islet transplantation closer to routine clinical consideration, pharmacologic immunosuppression is in fact detrimental to the long-term survival of islet transplants, necessitating a local form of immunosuppression that is adequate to control the rejection of the transplant without affecting the host immune response at a systemic level.

For diabetes, the choice of approach depends on the very nature of the underlying defect. In type 1, the objective is to educate the host immune system to eliminate autoreactive T cells before the clinical onset of the disease or to re-

Table 1. General Characteristics of Gene Delivery Vehicles

Vector type	Advantages	Disadvantages
VIRAL		
Adenovirus	Choice vector for pilot proof-of-principle experiments; high titers easily obtained; almost all cells and tissues are transducible; and cell retargeting is possible.	Immunogenic in vivo; nonstable transduction.
Adeno-associated virus (AAV)	Site-specific, stable integration achievable; almost absent immunogenicity; and many cell types transducible.	Time for transgene expression can be days.
Moloney murine leukemia virus (MMLV)-based retrovirus	Stably integrating vector in rapidly dividing cells; cell-type retargeting possible; and good titers are obtainable.	Subject to chromosomal position-effect sensitivity of, as well as methylation and cytokine effects on, gene expression.
Lentivirus	Nonimmunogenic, stably integrating; choice vector for nondividing, noncycling cells; good titers obtainable; data support absence of replication-competent-recombinant vector particles in stocks.	Clinical safety concerns with human immunodeficiency virus (HIV)-1-based vectors.
Herpes simplex type-1 virus	Large genome available for multiple large size cistrons; good persistence in many cell types; and cell-type retargeting possible.	Inherent toxicity.
NONVIRAL		
Plasmid DNA	Easy to engineer, grow, and purify; and multicistronic variants are easy to engineer.	Poor persistence, nonspecific cell targeting, and poor dissue diffusion.
Cationic liposome	Easy to manipulate to deliver plasmid DNA to almost all cells and tissue. Nonimmunogenic; cell-type nonspecific, and cell-type retargeting possible.	Poor control of diffusion kinetics.
Peptide fusion domains	Many cell-types transducible; high-level protein/peptide import; intact proteins/peptides delivered; not subject to gene regulation; targeting of specific proteins possible; high-level peptide production easily achievable; and no reported immunogenicity.	Short half life; subject to proteolytic degradation; and large amounts require some time to generate.

place the lost β cells with either intact islets or surrogate β cells. In type 2 diabetes, the objective is to initially restore metabolic control by regulating fuel metabolism, insulin sensitivity and production, and glucose uptake.

In general, there are two strategies that can be employed to generate a cell or gene drug: ex vivo engineering or in vivo delivery. In the ex vivo approach, a cell that will be used as a transgene factory is isolated from the host and manipulated in the laboratory. This may or may not involve cell expansion.

Most gene therapy strategies employ this approach. The in vivo interventions are more difficult because the targeting of the gene delivery vehicle becomes an issue and is currently difficult to achieve, unless the targeted site is where the correction is being made and is easily accessible.

Many of the approaches for type 1 diabetes involve gene transfer to cells ex vivo. Consequently, one must take into the account the nature of the cell to employ an appropriate delivery vector. The primary target cell type has been

the β cell in the form of intact islets for subsequent transplantation and immune cells of different types, most commonly the antigen-presenting cells or T lymphocytes. The in vivo approaches could involve targeting to the pancreas or organs and sites involved in immune stimulation, such as the thymus and the peripheral lymphoid organs.

To introduce a therapeutic transgene to β cells ex vivo, the delivery vehicle must be able to transduce these almost nondividing cells and to remain in the cell for its entire lifetime. The most

Table 2. Potential Intervention Strategies

Type 1 Diabetes Mellitus	
Gene Therapy	
<i>Facilitation of islet transplantation</i>	
<ul style="list-style-type: none"> • Ex vivo transduction with vectors encoding immunoregulatory transgenes (co-stimulation blockade, cytokine antagonists, and apoptosis inhibitors). • Infusion of antiapoptotic/immunoregulatory peptides into transplant recipients pre-transplantation and post-transplantation. • In vivo administration to transplantation site of vectors encoding trophic factors for islets. 	
<i>Tolerance induction</i>	
<ul style="list-style-type: none"> • In vivo immunomodulation using vectors encoding immunosuppressive/tolerogenic transgenes. • In vivo administration of vectors encoding putative autoantigens. 	
Cell Therapy	
<i>Surrogate β cells</i>	
<ul style="list-style-type: none"> • Embryonic stem cells differentiated into cells with β cell characteristics. • In vivo administration of vectors encoding genes promoting β cell phenotype to pancreatic/hepatic cells. • Expansion of progenitor cells ex vivo. • Therapeutic cloning technology. 	
Type 2 Diabetes Mellitus	
Gene Therapy	
<i>Prevention of insulin resistance</i>	
<ul style="list-style-type: none"> • Transduction of adipose and/or muscle with vectors encoding insulin-sensitizing genes and proteins/peptides. • Transduction of the liver with vectors encoding gluconeogenic-regulatory genes. • Transduction of peripheral, insulin-sensitive tissues with vectors encoding antagonists to cytokines inducing insulin-insensitivity. 	
<i>Anti-obesity strategies</i>	
<ul style="list-style-type: none"> • Targeting the hypothalamic-adipose axis (i.e., leptin, neuropeptide Y, and satiety factors). • Transduction of adipose with vectors encoding anti-lipolytic genes. 	
Cell Therapy	
<i>Surrogate β cells</i>	
<ul style="list-style-type: none"> • Embryonic stem cells differentiated into cells with β cell characteristics. • In vivo administration of vectors encoding genes promoting β cell phenotype to pancreatic/hepatic cells. • Expansion of progenitor cells ex vivo. • Therapeutic cloning technology. 	

versatile delivery vehicles are replication-deficient viral vectors, and Table 1 lists their advantages and disadvantages. In subsequent sections, their utility in various experimental models of type 1 diabetes gene therapy will be discussed. For peripherally derived antigen-presenting cells, the require-

ment for long-term expression may not be as stringent as for the β cells, primarily because the source of these cells (i.e., peripheral blood) is easily accessible. In this instance, novel approaches in addition to viral gene delivery can be employed. An example is the newly discovered peptide delivery vectors

Table 3. Current Approaches for Type 1 Diabetes Gene and Cell Therapy

Gene Vectors that Transduce Islets (with References)	Genes that Promote Islet Allograft/ Xenograft Survival In Vitro and In Vivo and/or β Cell Survival in Culture	Other Gene/Cell Therapy Approaches to Prevent/Abrogate Autoimmunity and/or Promote Islet Allograft/Xenograft Survival
Plasmid DNA (173,193–195) Adenovirus (196–209) AAV (210–219) MMLV-based retrovirus (220) Lentivirus (221–223) Herpes simplex virus (224,225) Cationic liposomes (194,195,204) Peptide fusion domains (52,53,56)	Anti-apoptotic genes bcl-2 (214,216,224–226) bcl-x _L (52,227) heme oxygenase-1 (228–230) dominant negative protein kinase C delta (231) dominant negative MyD88 (232) IGF-I (233) I κ B α super-repressor (209) Hsp70 (234) A20 (205) PEA-15 (52) catalase (235,236) manganese superoxide dismutase (237,238) Cytokines IL-4 (221) (although one report demonstrated no protection) (196) IL-1 receptor antagonist protein (208) IL-12p40 (197) viral IL-10 (198,239) IL-10 (240) (one report did not show protection) (196) TGF β (240) (one report showed negative results) (241) Immunoregulatory genes Indoleamine 2,3-dioxygenase (215) CTLA-4Ig (173) Fas ligand (199) (although in several reports, the Fas ligand was not protective) (242) adenoviral E3 genes (200)	Bone marrow transplantation/ chimerism induction (178,243–253) Antigen-presenting cell (APC) transfer class I MHC (163) autologous dendritic cell (DC) transfer (254) Co-stimulation blockade soluble ICAM-1-Ig (255) CTLA-4Ig (256–260) OX40Ig (259) Cytokines IL-10 (239,261–263) IL-4 (262,264) soluble IFN γ receptor (187,265) TGF β (266) Autoantigen transfer glutamic acid decarboxylase (GAD) (267) Others adenovirus E3 proteins (268)F orally administered putative autoantigens (insulin and GAD) (269–271) CD152 (272)

that can introduce proteins in their native forms across the cell and the nuclear membrane (53,57,58). These peptide-protein conjugates can be generated quite readily and have been shown to transduce many cell types in culture, including islet β cells (56).

However, in vivo gene delivery is more challenging and will require appropriate targeting. The direct injection of adenoviral vectors into the pancreas of rats has been attempted, as has plas-

mid DNA and adenoviral gene delivery to the thymus (59). Whether these approaches can become more efficient or clinically useful remains to be explored.

Type 2 diabetes, on the other hand, may be better suited for in vivo gene delivery at either the sites of metabolic defects or at sites where the biochemistry can be manipulated to restore normal glucose levels. For example, because insulin action on glucose metabolism is mainly on skeletal mus-

cle and adipocytes, one could target large muscle groups or sites of fat deposit with viral vectors (Table 1) to improve insulin sensitivity or to substitute skeletal muscle as a site of increased glucose uptake where other sites are defective. This approach can entail the use of direct injection of viral vectors or, as recently identified (60,61), injection of skeletal muscle stem cells that, once obtained from an autologous host, could be modified ex vivo with viral

gene vectors, expanded, and transplanted back into a large muscle group. However, pancreatic defects will probably require either direct injections into the pancreatic vasculature or islet/surrogate β cell transplantation. Whatever the defect, type 2 diabetes gene therapeutics will most likely be patient-specific because the defects among the patients are not expected to be identical.

Table 2 lists the general strategies aimed at therapeutic or prophylactic end points. Several vectors and transgenes, used to confer immune privilege and to bolster intrinsic defenses of the β cell, are listed in Table 3. Note that all of the studies we will describe have been performed in human or rodent islet/ β cell cultures or rodents *in vivo*. The results have been mixed. To date, there is no single transgene that has conferred significant long-term protection to transplanted islets in allogeneic diabetic rodent recipients. Moreover, although monogenic gene transfer to human or rodent islets has thus far been unable to prevent autoimmune rejection, as evidenced in rodent models of autoimmune diabetes, it is possible that multigenic gene transfer, which combines the properties of two or more transgenes in protecting islets at multiple levels or uses two or more vectors each encoding different genes, may succeed. Additionally, not all "immunoregulatory" genes have been tested. In the absence of these data, it would be unwise to view the transplantation of gene-engineered islets expressing multiple immunoregulatory genes as unfeasible or without potential. These studies must be carried out before reaching any general conclusion.

In parallel with the transplantation of gene-engineered islets, several attempts have been made to generate surrogate β cells from rodent or human progenitors. The most popular approach has been to convert hepatocytes or hepatocyte cell lines into glucose-sensitive insulin-producing cells for the purpose of transplantation. Direct *in vivo* administration of such vectors has also been attempted. Adenovirus has been the choice vector in these strategies, and several transgenes have been tested in the context of putative glucose-sensitive promoter elements.

Hepatocytes are particularly attractive because they can easily engraft in the liver, and because they possess glucose-sensing molecules identical to the pancreas [e.g., GLUT2 and glucokinase (GK)]. Furthermore, one can exploit several hepatocyte gene promoters that are sensitive to glucose to engineer insulin transgenes to be glucose concentration-sensitive. The disadvantages of hepatocytes, however, lie in their inability to secrete the insulin in a glucose-responsive fashion and their lack of PC2 and PC3 proconvertases and carboxypeptidase H, required to process proinsulin to its mature form (62,63). Despite these limitations, Groskreutz et al. (64) were able to engineer human proinsulin to contain furin cleavage sites at the natural sites of proinsulin processing. Furin is a ubiquitous protease that is also found in hepatocytes. This form of proinsulin was properly processed into active and mature insulin in transfected human kidney cells and rat primary myoblasts and possessed insulin receptor binding and activation activity similar to native insulin. Recent efforts have confirmed that hepatocytes are equally efficient at producing and processing this furin site-modified proinsulin into a mature, bioactive form (65). The need to express proinsulin in a glucose-dependent manner compelled Mitanchez et al. (66) to engineer transgenic mice expressing the human proinsulin cDNA or the furin cleavage site variant under the transcriptional control of the glucose-responsive liver isoform pyruvate kinase (LPK) promoter-enhancer elements. Insulin was expressed in the liver, kidney, and gut of these transgenic mice, and immunoreactive C-peptide was readily detectable in serum. Moreover, its levels were sensitive to levels of carbohydrates (66). More studies on this system using adenoviral gene transfer to the liver have recently been reported by Thule et al. (67,68), who have shown its potential in a streptozotocin-treated rat model. Very recently, Lee and colleagues (69) were able to achieve the complete restoration of normoglycemia in diabetic rats following recombinant adeno-associated virus (rAAV) gene transfer of a single-chain insulin transgene under the control of the LPK pro-

moter. Additionally, they obtained the same success in non-obese diabetic (NOD) mice. While these data are very compelling, the kinetics of changes in serum glucose concentration are not in harmony with the known kinetics of transcriptional activation of the LPK promoter. Furthermore, insulin secretion by hepatocytes is not glucose-regulated. In a related approach, Chen et al. (70,71) demonstrated in hepatoma cell culture that adenoviral gene transfer of insulin driven by the glucose-6-phosphatase promoter resulted in glucose-responsive, self-limiting insulin production, although these data are not clear on whether this approach can be successful in vivo.

Despite these promising approaches, much more work is needed to make hepatocytes into fully surrogate β cells. First, their response to glucose is not as rapid as that found in β cells. Second, the liver-specific glucose-sensitive promoters have elements that respond to hormonal and metabolic signals, which can impede, attenuate, or abrogate the desired objective of tight glucose regulation. For example, instances of hyperglucagonemia, which are to be expected in the absence of functional endogenous β cells in diabetics, will most likely attenuate or repress the LPK promoter and other promoters such as GK (72–74). Third, glucose-dependent transactivation of the LPK promoter requires the GK-dependent phosphorylation of glucose, an activity that is insulin-dependent (66). Other promoters have been suggested, such as phosphoenolcarboxykinase (PEPCK), but this promoter is activated by glucagon and inhibited by insulin, which may not result in the desired kinetics of physiological glucoregulation (75,76). It is possible that a combination of promoter elements from different glucose-responsive hepatic genes may be required to create an optimal synthetic promoter to drive hepatic insulin expression in a true glucose-sensitive fashion.

In addition to hepatocytes, one of the earliest approaches involved the genetic modification of an adrenocorticotrophic hormone (ACTH)-secreting neuroendocrine cell line (AtT20) to secrete human proinsulin where proinsulin expression was under the control

of a viral promoter (77). The advantage of these cells was they shared some features with β cells; namely, their possession of secretory granules and the expression of the PC2 and PC3 proconvertases and carboxypeptidase H, which cleave proinsulin to its mature form (78). Further manipulations of the cell line resulted in derivatives that expressed GK and GLUT2, the high Km glucose transporter of β cells. These cells were able to secrete insulin in a glucose concentration-dependent manner and responded to insulin secretagogues potentiated by glucose, which are features that are inherent in β cells (79). Despite this, the basal insulin release by these cells is higher than that of β cells. Another concern in the use of these cell lines is the effect of GLUT2, which is permissive for the secretion of ACTH and possibly other pro-opiomelanocortin-derived hormones in a glucose-dependent manner (66), potentially leading to iatrogenic Cushing's syndrome. Furthermore, the cytomegalovirus promoter may become inactive in vivo (80).

More recently, tissue-specific promoters have been exploited to engineer cells to express insulin in cells that are not targets of autoimmune destruction. Lipes et al. (81,82) have expressed insulin in the anterior pituitary of NOD mice under the control of the pro-opiomelanocortin promoter. Insulin was expressed, stored in secretory granules, and exhibited regulated secretion. Moreover, the transplantation of transgenic anterior pituitary tissue to NOD mice was able to partially restore normoglycemia with no signs of immune rejection (81,82). However, it was unclear if insulin secretion was glucose concentration-dependent in these cells. Very recently, an ingenious approach harnessing intestinal K-cells as surrogate glucose-responsive insulin producers was demonstrated. In this approach, transgenic mice expressing human insulin under the control of the gastrointestinal inhibitory peptide (GIP) promoter were generated. These mice expressed and secreted insulin from intestinal K cells in which the GIP promoter is active. Insulin secretion in these mice was glucose-responsive and maintained following streptozotocin treatment, indicating that the K cells

were spared the effects of streptozotocin (83). These data suggest that it may be feasible to target the intestinal cells with vectors encoding the GIP-insulin transgene or by ex vivo engineering of intestinal cells in which glucose-sensitive promoters are active driving insulin expression.

EXPANDING β CELLS OR β CELL SURROGATES

The considerable genetic manipulations that are required to convert non- β cells into efficient glucose-sensing, insulin-secreting cells have led other investigators to consider means of expanding adult or neonatal β cells or of harnessing the developmental potential of islet precursor cells to commit them to the β cell lineage. Even disregarding possible ethical concerns, the successful isolation of purified islet stem cells obtained from the fetus or neonate has remained elusive. Where precursor cells have been identified, commitment to β cells and insulin production has not always been consistent (84–88). Recent data indicate that embryonal stem cells can also assume insulin-producing properties (89). If a stable β cell-like phenotype can be maintained for these cells in culture and following transplantation, then they could be a source of renewable surrogates for β cells following manipulations to expand them.

Despite the current controversy and the serious ethical issues raised by cloning technology, it is likely that therapeutic cloning, under strict and defined conditions, will find its place in stem cell therapies (90–92). In this regard, a possible means of propagating β cells or progenitors, while avoiding the complications involved with the immune response, could entail nuclear transfer approaches. In this method, the nucleus (containing donor DNA) from somatic cells of a patient may be transferred into an enucleated embryonal stem cell that can be expanded into an appropriate β cell lineage. However, the issue of autoimmunity will remain. While this is highly speculative at present, the rapid pace of basic work in this area, despite restrictions, will likely yield insight into such manipulations.

The immortalization of islet cells with a β cell phenotype has been attempted and successfully achieved. Insulin production, however, seems to be linked to the terminal differentiation of the cell, an event that is normally reached with growth arrest. This problem has so far limited the utility of cell immortalization. This approach also carries with it the troublesome possibility of oncogenic transformation (93–96).

Although still controversial, there are data indicating that mature human β cells can be induced to replicate under the effects of hepatocyte growth factor (HGF) (97–99). However, the limitation of this approach rests on the loss of the differentiation of the induced β cell, along with a substantial decrease in insulin production (100). Conditional replication of nonhuman β cells has been achieved by placing the simian virus 40 (SV40) T antigen under the control of an inducible promoter (94). In these studies, β cells were able to replicate and to maintain differentiated function under inducible conditions. No data exist on whether such an approach is feasible in human β cells.

The propagation of islet precursor cells with subsequent genetic manipulation to commit them to the β cell lineage and ultimately to β cells has also been considered (88,101). For this approach to become feasible, however, a more complete understanding of the hierarchy of master regulatory transcriptional genes is required. Nevertheless, some progress has been made following the discovery that the absence of pancreatic duodenal homeobox (PDX)-1 (also known as IDX-1, IPF-1, STF-1, and IUF-1), a homeodomain transcription factor, results in pancreatic agenesis (102). In addition to differentiation of primitive pancreatic epithelium, PDX-1 appears to be important in glucose-sensitive transcriptional transactivation of the insulin gene and GLUT2, GK, and somatostatin (103–106). Depending on the cell type, PDX-1 overexpression can impart onto it a β cell or a β cell-like phenotype (102,107,108). Indeed, Ferber et al. (109) demonstrated that the adenoviral gene transfer of a PDX-1 gene into the liver resulted in insulin-expressing cells, although it was unclear whether these cells were

glucose-sensitive (109).

The transfer of combinations of genes encoding soluble and intracellular differentiation factors could become feasible once their precise role in the pathway of commitment and differentiation becomes clearer. However, β cells have a limited lifespan *in vitro*. To what extent apoptosis or senescence play a role in this is uncertain. Nonetheless, a better understanding of cell cycle control in β cells or neonatal islet cells could lead to the discovery of molecules that could be exploited, in a conditional manner, to promote growth *in vivo* and maintenance or extension of lifespan, both *in vitro* and *in vivo*. Possible means include the transfer of cyclin-dependent kinases, pro-replication and mitotic factors, and/or telomerase to promote expanded cell lifespan, all under regulatable promoters. Such an approach could achieve the expansion of semi-committed or fully committed islet precursor cells or early β cells. Combined with xenogeneic donor manipulation, these interventions could provide an almost limitless supply of β cells for transplantation.

ISLET OR SURROGATE β CELL TRANSPLANTATION

Whatever the identity of the insulin-replacement cells (intact islets, *ex vivo*-generated β cells, or surrogate β cells), unless they are of autologous origin, they will need a defense against allogeneic, xenogeneic, and autoimmune attack. There are two levels at which these defenses can be deployed: (i) extracellularly, by the production and secretion of immunosuppressive and/or anti-apoptotic gene products, and (ii) intracellularly, where anti-apoptotic protein production could prevent intracellular pathways triggered by extracellular death ligands. Many approaches have been tested, and all are listed in Table 3.

APPROACHES FOR TYPE 2 DIABETES

Being the predominant risk factor for insulin resistance, obesity is an important target by which the eventual

progression to type 2 diabetes can be prevented. Obesity is, itself, a multifactorial syndrome caused by genetic and environmental factors. While the simple solutions to obesity are to consume in moderation, avoiding high-calorie foods on a long-term basis, and physical exercise for morbidly obese individuals or in those noncompliant to dietary regimens, gene drugs may be the alternative. The primary objective in these gene drugs would be to reduce satiety levels and to regulate the amount of fat. One of the principal regulators of body fat and a fuel sensor is leptin.

Leptin was initially identified as the gene whose mutated variants resulted in the obese phenotype of obese (ob/ob) mice. Leptin encodes a secreted protein with weight-reducing, satiety-suppressive, and insulin- and glucose-lowering effects (110–114). In a small group of morbidly obese humans, the leptin gene was also found to be mutated, but evidence suggests that almost all forms of obesity in humans are not due to leptin defects (115–118). A series of studies demonstrated that viral gene transfer of leptin to rodent models of obesity was effective in accelerating weight reduction, decreased adipocyte and fat deposits, enhanced fatty acid oxidation, and lowered expression of lipogenic enzymes (119–122). Moreover, a single intramuscular injection of adeno-associated virus (AAV) encoding leptin into ob/ob mice was able to achieve a long-term normalization of metabolic abnormalities, such as impaired glucose tolerance, insulin resistance, hyperglycemia, and lethargy (123). In instances where no leptin receptor defects are detected, leptin gene transfer may prove beneficial, at least to prevent or delay the onset of obesity as a preventative measure against obesity-induced insulin resistance, which precedes type 2 diabetes. Leptin, acting at the level of the hypothalamus, controls a very complex satiety system and, in this regard, leptin expression could be targeted to tissues in which feedback to the hypothalamus and the pituitary occurs in response to feeding.

The observation that the ratio of leptin in the cerebrospinal fluid to serum in obese individuals is higher compared to normal controls suggests that most

obese individuals do not have defects in the leptin gene and are resistant to its effects either because of a leptin receptor or leptin transport defect (124). These data indicate that alternative strategies aimed at bypassing the hypothalamic targets of leptin thought to be resistant to leptin are necessary and, in this regard, a better objective could be the reestablishment of insulin sensitivity in obese individuals. Identically, such an objective is desired in non-obese insulin-resistant individuals.

Reestablishment of Insulin Sensitivity

Insulin insensitivity is one of the main characteristics of type 2 diabetics and is associated with increased β cell insulin secretion along with hyperinsulinemia. Eventually, β cell exhaustion is thought to occur, which results in overt hyperglycemia. Consequently,

insulin resistance can be thought of as two impairments whose temporal relationship is not fully clear: peripheral insulin insensitivity with β cell compensation, culminating in β cell exhaustion (125–127). Whatever the temporal order, the reestablishment of peripheral insulin sensitivity is paramount to prevent β cell exhaustion; therefore, strategies aimed at bypassing defects in insulin sensitivity in peripheral tissues (skeletal muscle, adipose, and liver) or to promote glucose uptake and utilization in the periphery must be considered as a first approach in gene therapy strategies.

The major glucose transporter in the periphery is GLUT-4, and studies with transgenic mice indicate that GLUT-4 expression in peripheral tissues could be one way to prevent or ameliorate insulin resistance. Leptin-resistant, diabetic (db/db) mice (whose genetic defect is in the leptin receptor

gene), transgenic for the human GLUT-4 transgene, exhibited reduced fasting and nonfasting plasma glucose levels, improved glucose disposal, and insulin-sensitive, glucose-responsive translocation of GLUT-4 to the cell surface (128). In high-fat fed mice where GLUT-4 expression was driven by the fatty acid binding protein aP2 promoter, an adipocyte-specific promoter, a slight improvement in glucose tolerance and increased transport in adipocytes and adipocyte hyperplasia was observed. That the improvement of glucose tolerance was not major was thought to be due to the absence of the GLUT-4 transgene in skeletal muscle, the major tissue of glucose utilization (129,130). Improvements in glucose tolerance and insulin sensitivity were achieved in transgenic mice where GLUT-4 was driven by skeletal muscle-specific promoters. Hansen et al. (131) demonstrated that glucose

transport into skeletal muscle was significantly increased and insulin-sensitivity compared to nontransgenic controls, and, moreover, glycolysis and glycogen synthesis were enhanced in isolated skeletal muscle (131). Additionally, in another transgenic mouse model deficient in GLUT-4, in which GLUT-4 was expressed under the control of the myosin light-chain promoter, the uptake of a synthetic glucose analogue and the actions of insulin were enhanced and restored to normal levels *in vivo* (132). In a heterozygous GLUT-4 knock-out mouse model, complementation of the gene in skeletal muscle was able to restore insulin sensitivity and prevented a diabetic phenotype (133).

Overexpression of the insulin recep-

tor along with GLUT-4 and insulin receptor-downstream signal transducers such as the insulin receptor substrate (IRS) proteins could be another means of restoring normal insulin sensitivity. The rationale for this derives from studies in homozygous or heterozygous transgenic mice deficient for the insulin receptor, IRS-1 and IRS-2 (134). Muscle-specific insulin receptor knock-out mice are insensitive to insulin in muscle but not in liver or adipose, whereas β cell-specific insulin receptor knock-out mice exhibit an impaired response to glucose, yet are sensitive to insulin in the periphery. However, IRS-1 and IRS-2 knock-out mice are hyperinsulinemic with a mild to severe diabetes where the insulin insensitivity is at the level of muscle and adipose cells in

IRS-1 knock-outs and at the level of liver in IRS-2 knock-outs (134). Ueki et al. (135) demonstrated an almost complete restoration of normal glucose sensitivity in IRS-1-deficient mice injected systemically with an adenovirus encoding IRS-1. Other genes that have been transferred to skeletal muscle include GK using an adenoviral vector *in vitro*. In that study, an increase in glucose uptake was noted, independently of insulin stimulation, as well as a glucose concentration-dependent accumulation of glucose in skeletal muscle (136). In a follow-up study, the same group demonstrated that adenoviral gene transfer of GK to hepatocytes from Zucker diabetic rats (one rodent model of obesity and insulin resistance) was able to promote normal glucose

Figure 2. Gene and cell therapy strategies to promote islet allograft/xenograft survival and/or prevent diabetes. Several strategies can be employed alone or in combination: (i) gene transfer of immunoregulatory molecules to autologous cells that can modulate immunoregulatory networks (such as dendritic cells); (ii) gene transfer of cytoprotective genes to allogeneic or xenogeneic islets that will be subsequently transplanted; (iii) protection of β cell mass and function during the isolation phase of islets; (iv) gene transfer of factors that promote a β cell phenotype to β cell progenitors or surrogate cells; (v) and the direct transfer of vectors encoding immunoregulatory molecules into a susceptible or recent-onset patient.

uptake and conversion to glycogen. More recently, *in vivo*, transduction of newborn rat skeletal muscle with adenovirus-GK achieved an increase in glucose uptake and ameliorated total body glucose tolerance (137). Finally, Etgen et al. (138) used an adenovirus encoding human protein kinase C-zeta to transduce rat skeletal muscle *in vivo*. Protein kinase C-zeta is believed to be a constituent of the insulin receptor signaling pathway, and, in that study, the investigators observed enhanced glucose uptake in the skeletal muscle of Zucker rats (138).

Thiazolidinediones are a class of glucose-lowering agents that act to increase insulin sensitivity. Their action is at the level of peroxisome proliferator-activated receptor γ (PPAR γ), a member of the nuclear transcription family of proteins, although PPAR γ -independent activities have also been observed (127). Intriguingly, heterozygous knock-out mice for PPAR γ do not develop insulin resistance, but, in contrast, display enhanced insulin sensitivity (127). This and other observations suggest that the genetic inhibition of PPAR γ activity could prevent insulin resistance, and an approach could be the targeting of dominant-negative variants of PPAR γ to insulin-sensitive tissues such as skeletal muscle or adipose.

The activity of cytokines on insulin resistance has been well documented. Tumor necrosis factor- α (TNF α) in particular induces insulin resistance *in vitro* and *in vivo*. Cheung et al. (139) demonstrated that systemic gene transfer of a soluble TNF receptor-Fc transgene using an adenoviral vector in Zucker rats enhanced the peripheral insulin sensitivity and suppressed hepatic glucose output (139). It has been suggested that other cytokines such as interleukin (IL)-1 β and IL-6 might be involved in insulin resistance, and targeting their binding to receptors using soluble receptor or antagonist decoy strategy might be beneficial.

Insulin Replacement

Where peripheral insulin sensitivity is normal and the defect lies at the level of the β cell, β cell augmentation or replacement strategies may overcome the

endogenous defect(s). An approach could be the transplantation of allogeneic islets or the engineering of surrogate β cells. Additionally, the identification of β stem or progenitor cells is also an area that is promising (140–145), especially in light of recent discoveries that bone marrow-derived cells can be made to differentiate into neuronal and muscular cells (146). In the event that the donor is allogeneic, some degree of immunosuppression will be required, and approaches for these strategies have been discussed in an earlier section.

EDUCATING THE IMMUNE SYSTEM

Type 1 diabetes mellitus is perhaps one of several autoimmune diseases where it may be possible to induce tolerance. The autoimmunity is directed

against β cells, which suggests that one or more molecules expressed by β cells may be employed to tame the immune system. There are some lines of evidence that support the concept that insulin may be an important β cell protein to which developing T cells failed to be educated. First, are the genetic data, where the insulin locus has been linked to type 1 diabetes (147–151). The second line of evidence in support of insulin as an important autoantigen is the demonstration of circulating autoantibodies and, more recently, an insulin-peptide-reactive cytotoxic T cell clone that was isolated from the islet infiltrate of young NOD mice (152, 153). While insulin remains a strong contender, there is very good evidence in support of other putative antigens such as glutamic acid decarboxylase (GAD) and IA-2 (1,2). These other proteins, however, are expressed in a variety of other nonendocrine cells and

tissues, and it is unclear how they may specifically contribute in type 1 diabetes autoreactivity (154).

If, indeed, type 1 diabetes is the result of a failure to delete lymphocytes in the thymus that would normally recognize β cell antigens, then it is reasonable to propose that thymic over-expression of putative autoantigens in early life, before the onset of type 1 diabetes in individuals deemed at high risk, could prevent the disease by genetic screening criteria. This line of reasoning was initially adopted for studying diabetes in the NOD mouse as well as in the BioBreeding (BB) rat, two rodent models whose diabetes-related immunopathology is considered to be quite similar to that in humans. In NOD mice, the expression of a proinsulin transgene controlled by the MHC class II promoter, resulting in the intrathymic expression of insulin, was able to prevent diabetes (155). This

outcome was also achieved in BB rats and in young NOD mice by the intrathymic injection of islet extracts (156–160). In these studies, Posselt et al. (156–158) suggested that tolerance occurred as a consequence of the deletion of islet-reactive thymocytes. The intrathymic injection of insulin B-chain or the 65-kDa variant of GAD into young NOD mice was also able to suppress type 1 diabetes onset (161). However, it is unclear whether this approach will work in post-pubescent humans because of a lack of knowledge concerning thymic delivery. More important is the persistence of the vector for the entire lifetime of the individual and the risks associated with anti-vector response in vivo, depending on the choice of vector, although recent results suggest that thymic gene delivery using adenoviral vectors is possible and can also result in tolerance to the virus-encoded antigens (162).

A second possibility could be to identify the precursors to thymic antigen-presenting cells and to expand them ex vivo concurrent with engineering them to express one or more autoantigens using several gene delivery vectors. Upon introduction into the patient, these cells are expected to migrate to the thymus to express the autoantigen(s). The caveat to this approach is that this cell therapy will be patient-specific and require the isolation of bone marrow cells from which thymic antigen-presenting cell (APC) precursors could be propagated. There is encouraging evidence that this can be clinically feasible. A third and exciting possibility is to take advantage of peripherally derived APCs that can be engineered to possess tolerogenic properties, acting peripherally and eliminating or silencing circulating autoreactive T cells. In fact, we and others have demonstrated that this approach can facilitate allogeneic cardiac and islet allograft survival, and a minor modification of this approach could be applied to tolerance to autoantigens (163–166).

Full activation of T lymphocytes requires a second signal in addition to the interaction between the T cell receptor (TCR) and the peptide/major histocompatibility complex (MHC) class II complex on an APC (13). This second signal, acting through the CD28 molecule at the surface of T lymphocytes, can be provided by the B7 molecules, B7-1 (CD80) and B7-2 (CD86), which are expressed at the surface of APCs. Blockade of CD28-B7 interaction leads to T cell anergy or apoptosis (167). The fusion, CTLA-4Ig, effectively acts as a decoy for B7 molecules and prevents the B7-CD28 interaction. Several studies illustrated in Table 3 demonstrate the feasibility of the gene transfer of this agent to prevent and modulate type 1 diabetes (168–178).

THE CHOICE OF DENDRITIC CELLS AS EDUCATORS

Although considered potent immunostimulators, dendritic cells (DCs) have recently been shown to possess tolerogenic characteristics under defined conditions. DC tolerogenicity,

manifested as the suppression of T cell activation, has been documented in tumor-, allo-, and auto-immunity (179). The conditions that can yield tolerogenic DCs include ultraviolet irradiation and exposure to CTLA-4Ig, transforming growth factor (TGF) β , or IL-10 (180). How a tolerogenic DC acts to suppress immunoreactivity is not completely understood, but it may involve anergy, a shift to TH2-type responses, apoptosis, or the induction of regulatory cells (179). Myeloid DCs have been genetically modified using adenoviral and retroviral vectors encoding CTLA-4Ig, TGF β , and IL-10 (180). CTLA-4Ig-expressing DC significantly prolong allograft survival, can induce alloantigen-specific T cell hyporesponsiveness, and display enhanced survival in nonimmunosuppressed, allogeneic hosts (180). The *in vivo* presentation of alloantigens by donor or recipient DCs in the absence of co-stimulation, along with the local production of immunosuppressive molecules such as TGF β , could likely promote the inhibition of anti-donor reactivity and promote tolerance induction with no major systemic immunosuppression. DCs engineered to express vIL-10 following retroviral gene transfer produce high levels of vIL-10 *in vitro*, exhibit marked reduction in cell surface MHC and co-stimulatory molecule expression, decrease T cell allostimulation, and promote the induction of T cell hyporesponsiveness (181). Genetically engineered DCs may be used to prevent islet allograft rejection because they are able to manipulate anti-donor and/or autoantigen immunoreactivity. Alternatively, if recent observations that show islet-specific molecule gene expression in peripheral lymphoid organs can be confirmed in APCs (182) such as bone marrow-derived DCs (unpublished observations), one can envision infusing engineered DCs that lack co-stimulatory capability but that express islet-specific genes (e.g., GAD65 or insulin) into prediabetic or early-onset diabetic patients, with the objective of inducing autoantigen-specific tolerance.

A related approach can be similar to that described by Zhang et al. (183), who have engineered DCs to express Fas ligand as a means of inducing tol-

erance (183). Peripheral blood mononuclear cells can be cultured *ex vivo*, and DCs can be propagated using a cytokine cocktail (184). These DCs, or their monocyte precursors, could be engineered with several different gene delivery vehicles, each of which alone or as a multi-cistronic construct could encode putative autotantigens along with proteins that can induce the apoptosis of T cells with TCR that recognize the over-expressed antigen peptides or could silence autoreactive T cells. One can envision, for example, DCs expressing GAD and Fas ligand or CTLA-4Ig. In the first instance, the T cells with TCR specific for GAD epitopes, upon reacting with the DCs, will encounter Fas ligand, and, as activated T cells expressing Fas, will be induced to undergo apoptosis. In the other instance, GAD-reactive T cells will be unable to receive the CD80, CD86 co-stimulatory signal, because of the CTLA-4Ig produced by the DCs and will therefore be unable to proliferate. Indeed, studies in allogeneic transplant models have demonstrated that DCs that are engineered to express TGF β or CTLA-4Ig can induce donor-specific hyporeactivity *in vitro* and can significantly prolong allogeneic heart transplants when injected *in vivo* (180,185). More recently, DCs have been treated *ex vivo* with oligodeoxynucleotide decoys to nuclear factor κ B (NF- κ B), an important maturational transcriptional mediator in DCs and, when injected into an allogeneic host, were able to prolong the survival of an allogeneic heart (166). It is likely that other transcriptional pathways in APCs could be exploited by decoy nucleotide strategies to present autoantigen in the absence of co-stimulatory signals or in the presence of death ligands to silence or kill autoreactive T cells.

IMMUNOREGULATORY VACCINES

Several studies have shown that the injection of DNA plasmid vectors encoding immunosuppressive genes, such as TGF β , and a soluble interferon (IFN) γ receptor into the muscle of NOD mice can reduce the incidence of

type 1 diabetes (186–188). The mechanism for protection is unclear but may involve the suppression of maturation of APCs in the periphery and the suppression of autoreactive T cell activity before and during insulinitis. Furthermore, the incomplete penetration of the protection could very well be due to the nature of the plasmid DNA vector and its low persistence, especially at the sites of injection. Another critical factor that may have influenced this exciting approach is the nature of the cells that take up the plasmid vector. It is possible that the immunosuppressive transgene was expressed in migratory APCs at the site of injection of the mice in which protection was achieved, which then could deliver the immunosuppressive protein to peripheral lymphoid organs. In the animals in which no protection was achieved, the vector may have been taken up by other cells such as skeletal muscle and fibroblasts, which, by their nonmigratory character, confined the expression of the immunosuppressive transgene locally at the site of injection. To enhance this approach, one could target vectors to APCs either by pseudotyping with envelopes engineered to possess ligands for APC-specific receptors or in cationic formulations into which these APC-specific proteins are conjugated. Bioballistic gene delivery may offer advantages for this approach over injection, as several studies indicate that Langerhans cells and migratory APCs are more efficiently engineered to express a transgene by gene gun delivery (189,190). Finally, in theory, one could also isolate Langerhans cell progenitors from a patient, expand them ex vivo, engineer them in a manner similar to that described above (i.e., multicistronic vectors encoding autoantigen, along with immunoregulatory gene products) and introduce them subcutaneously, where they will migrate to the peripheral lymph nodes to engage autoreactive T cells.

While manipulating APCs to express autoantigens in a tolerogenic context may be one means of promoting tolerance, an alternative method could involve the engineering of thymic APCs to express human leukocyte antigen (HLA) alleles that are not associat-

ed with type I diabetes. The expression of HLA DQ and DR alleles conferring susceptibility, for example, could be suppressed by antisense approaches, followed by the supplementation of the thymus with thymic APCs, engineered ex vivo to express nondiabetogenic class II HLA alleles. The rationale for this approach lies in the observations of Singer et al. (191), who have demonstrated that type 1 diabetes was prevented in transgenic NOD mice expressing a nondiabetogenic MHC class II molecule in place of the diabetogenic I-Ag7. We encapsulate all the approaches reviewed and proposed here in the cartoon in Figure 2.

GENES, CELLS, OR BOTH?

It is premature to make conclusive statements about the future clinical utility of gene therapy for disorders of glucose metabolism, especially diabetes mellitus. Despite the current priority shift from gene transfer to stem cells, more questions and concerns have been raised regarding stem cells compared to gene therapy approaches. In fact, a very recent report appears to refute many pivotal discoveries of putative insulin-producing stem cells (192) and outlines several critical tests that this area of investigation must pass before further consideration. Gene therapy, on the other hand, has passed the conceptual hurdles and has demonstrated strong potential for clinical translation, either as a means of facilitating islet transplantation and/or educating the immune system to accept allogeneic cells and tissues. More emphasis should be placed on the translational research in this realm, with a crucial evaluation of multigenic approaches in low-immunogenicity vectors. Ultimately, we predict that ex vivo engineering of cells will yield the first successful translation of promising animal studies into the clinic.

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