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ORIGINAL PAPER

The status of gene vectors for the treatment of diabetes

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Abstract Diabetes mellitus type 1 (DM1) represents one of the most obvious targets for successful treatment by gene transfer. The disease provides targets and methods for therapy that include suppression of autoimmunity, restoration of insulin responsiveness, functional replacement of pancreatic islets, and correction of vascular and nerve damage associated with prolonged hyperglycemia. The pathogenesis of DM1 is well understood and gene sequences are known that would support these various approaches for genetic intervention. However, a key limitation at present is the availability of efficient and reliable methods for delivery and sustained expression of the transferred DNA. Most genetic vectors are derived from viruses, and recent improvements in adenovirus-derived, lentivirus-derived, and adeno-associated virus-derived vectors suggest that these will have successful application to diabetes in the future.

Keywords Vectors · Lentivirus · Adenovirus · Adeno-assoicated virus · Retrovirus · Gene therapy

Introduction

The transfer of genetic material with therapeutic intent is the essence of gene therapy, and it requires three basic elements: (1) an understanding of the pathogenesis of a disease and a valid rationale supporting the proposed genetic treatment, (2) a genetic sequence which if transferred to cells could be therapeutic, and (3) the ability to deliver such a gene to the appropriate tissues with the level and duration of expression necessary for the therapeutic effect. These issues are usually summarized under "Points to Consider," among other technical and ethical considerations, within any proposal for such interventions by gene transfer [1].

Diabetes mellitus type 1 (DT1) is an ideal target for new treatment using gene transfer for the reason that DT1 is very well understood in terms of the pathogenesis of the disease. The rationale for genetic approaches to DT1 include four basic approaches: suppression of autoimmunity, replacement of a physiologic insulin response, replacement of islets per se, and correction of the vascular and nerve damage associated with the disease [2]. Genetic approaches have been proposed aimed at curtailment of the immune activation and islet cell destruction [3–6]. To accomplish specific protein replacement, insulin expression after gene transfer into animal or tissue models is an area of considerable interest [7–9]. This approach would actually by-pass the disease process and assist in replacement of pancreatic islet function. Genetic methods have been proposed to expand islets in vitro to facilitate islet transplantation [10]. The late complications due to vascular insufficiency and to neuropathy have potential molecular therapies that are already being addressed in non-human primate studies [11] as well as in gene transfer trials in humans [12–14]. In part, the richness of our understanding of the pathogenesis of DT1 creates a complexity that could actually diminish gene therapy approaches, since it is not clear which approaches should be pursued in DT1 treatment.

Would the restoration of insulin levels be therapeutic or will correction of the autoimmunity that underlies DT1 be necessary? Could, instead, attention to the vascular abnormalities associated with distal organ degeneration become a therapeutic approach separate from alterations in

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Table 1 Vectors used in gene therapy of diabetes

Data adapted from the NIH Office of BiotechnologyActivities website—http://www4.od.nih.gov/oba/rac/clinicaltrial.htm

Investigator	Project	Vector
R. Kuntz	Re-stenosis Gene Therapy	Liposomal cDNA Inducible nitric oxide
R. Crystal	Peripheral Vasc Ischemia	AdV5 VEGF
A. Ropper	Diabetic Neuropathy	Plasmid cDNA VEGF
D. Losordo	Diabetic Neuropathy	Plasmid cDNA VEGF
D. Cornblath	Diabetic Neuropathy	Ploxamer-DNA VEGF

the basic pathologenesis? As shown in Table 1, there have been five clinical trials approved for patients with diabetes, and all of these have addressed issues regarding late complications of diabetes, including re-stenosis of coronary arteries, peripheral vascular ischemia, and diabetic neuropathy. In these studies, the gene used for 'treatment' has been chosen to achieve improved vascular profusion, usually by means of vascular endothelial growth factor (VEGF). Thus, although the field of gene therapy for DT1 remains immature, it is growing rapidly [15].

However, before these approaches can be evaluated in patients, the methods of delivery of gene sequences must become available. Transfer of genetic information uses packaged gene sequences, either in the form of DNA or RNA, and these packages are called 'vectors'. A vector is any vehicle used for achieving adequate delivery of the therapeutic gene, and these can be complexed with synthetic polymers or lipids, can utilize natural virus constructions, or can be just naked DNA. The purpose of this chapter is not only to introduce the reader to the various approaches for gene therapy of DT1, but also to review the field of vector development.

The ideal gene vector

The features that are required for an ideal gene vector are basically those that would be necessary to produce the desired clinical effect. For certain goals, only a transient expression of a gene might be necessary, e.g., vaccines using recombinant viruses or DNA, but for most corrective treatment there must be continued gene expression and this must be under physiologic controls. These features are summarized in Table 2. For DT1, the ideal gene therapy vector would usually be one that maintains adequate expression of the transgene for the lifetime of the patient, has the ability to target the disease site, provide 'transgene space' sufficient for all the required transcription promoters or enhancers needed, and delivers the genetic payload in a safe manner. In this regard, the ideal vector would be one that would not produce mutations when integrated into host DNA and would not produce a product that would be immunogenic and target the transduced cells for immune attack. Finally, the vector should be relatively easy to produce in high concentration and do this using a method that is relatively inexpensive. If insulin replacement is the goal, the gene should have the capacity to encode a full length complementary DNA (cDNA) and have the capacity to encode promoters and/or enhancers which facilitate physiologic responses to changing glucose levels. As shown in Table 3, vectors that have been approved for use in clinical gene therapy trials range from recombinant viruses, to liposomes, plasmids, and complex polymers. Retroviruses and adenoviruses are the leading type of vectors that have been used to date. Adeno-associated viruses (AAV) are rapidly gaining ground because of their efficiencies in clinical use. Lentiviruses are a relatively new vector class that is generating considerable interest and which are just entering clinical evaluations.

Adenovirus vectors

Adenoviruses are double-strand DNA viruses in the *Adenoviridae* family and can enter a cell using surface fiber proteins that bind to cell receptors, usually the coxsackie-adenovirus receptor (CAR). Adenovirus infection is a transient one which results in rapid virus replication and destruction of the infected cell. Adenovirus uses genes such as E1A to activate subsequent genes that are necessary for its replication, especially a gene called E2, and E2 led to the discovery of the E2F family of host cell proteins which are important in cell cycle control [16]. Adenovirus type 12 was the first human virus demonstrated to induce tumors in animal models, but adenoviruses have not been associated with any human tumors. Nevertheless, this transforming

Table 2 Features of the ideal gene therapy vector

1.	Expression of transgene for lifetime of patient
2.	Ability of the vector to target the disease site
3.	Safety of the vector; lack of off-target effects
4.	Lack of integrational mutagenesis
5.	Lack of immunogenicity: innate and adaptive responses
6.	Capacity to encode full length cDNA
7.	Additional capacity to encode promoters and enhancers which facilitate physiologic expression of the transgene
8.	Easy and inexpensive to produce in the laboratory

Table 3 Vectors used in gene therapy

Vector	Total Protocols	Protocols 1999–04
Retrovirus	263	83
Adenovirus	258	172
Lipofection	85	10
AAV	25	21
Other virus	148	100
Others	183	148

Adapted from Alba et al. Gene Therapy 2005; Suppl 1:S18–27

activity of adenovirus has forced investigators to delete specific genes of the virus that could potentially be oncogenic or pathogenic. With the complete elimination of viral proteins necessary for early control of cell metabolism, the vector is unable to replicate and is a helper-dependent adenovirus, meaning it must be grown in the presence of certain proteins required for its replication [16].

The concept of vector production using helper function is central to all replication incompetent viral vectors, and has been essential for the development of gene transfer vectors from pathogenic viruses. This is best illustrated with adenovirus, and, as shown in Fig. 1, the candidate vector sequence is constructed from a wild virus sequence by deletion of several of the genes encoding the required proteins necessary for virus replication and assembly. But the vector sequence must contain a signal for packaging and this is usually located near the transgene(s) of interest in order for correct packaging of the viral vector. To replace the missing proteins required in transcription of the vector genes and assembly into new virions, tissue culture cells are used which have these 'missing' genes transferred into them in the form of plasmid DNA. Alternatively, another virus infection or a cell that constitutively expresses the missing genes can substitute for the deleted viral genes. When the plasmid DNA is constitutively expressed as a cell, this can be used as a packaging cell line for ease of vector production. If replication competent helper virus is used, then this must be removed from the final product. The final recombinant viral vector must then be shown to be replication non-competent by appropriate release tests. The structure of the viral vector, with its surface proteins, is maintained so that it can continue to target specific cells.

First-generation adenoviral vectors contain the whole viral genome with the exception of the E1 region [17], and these vectors cannot replicate but they can induce immune responses that are both innate and adaptive responses. A second-generation adenoviral vector removed E1, E3, and E2–E4 complex, and this vector provided 14 kb of increased cDNA capacity but could still induce immune responses. A further deletion of the adenovirus vectors that retain only the 5′ and 3′ inverted terminal repeats (ITRs) and the packaging signal from the wild type adenovirus has created the third generation of adenovirus vectors [18]. These so-called "gutless adenovirus vectors" have nearly complete removal of the original adenoviral genes and result in a helper-dependent vector that requires viral protein supplied in trans by the cell system.

The problem with adenovirus vectors is that they can induce initial innate immune responses that release proinflammatory cytokines and lead to complement activation. The innate immune responses induced by adenovirus derive from activation of Toll-like receptors and, with induction of complement activation and a hypercoagulability state, can be very toxic [19, 20]. The adverse effects of adenovirus after direct intravenous or intra-arterial injection of either macaques, baboons, or humans have included elevation of liver enzymes, alteration of coagulation factors and platelet counts, and even death [16, 19, 20–23].

With the development of the gutless adenoviral vectors, there is minimal immune reactivity of either an innate or an adaptive nature, and there is prolonged and stable cellular expression. These vectors have a capacity to hold up to

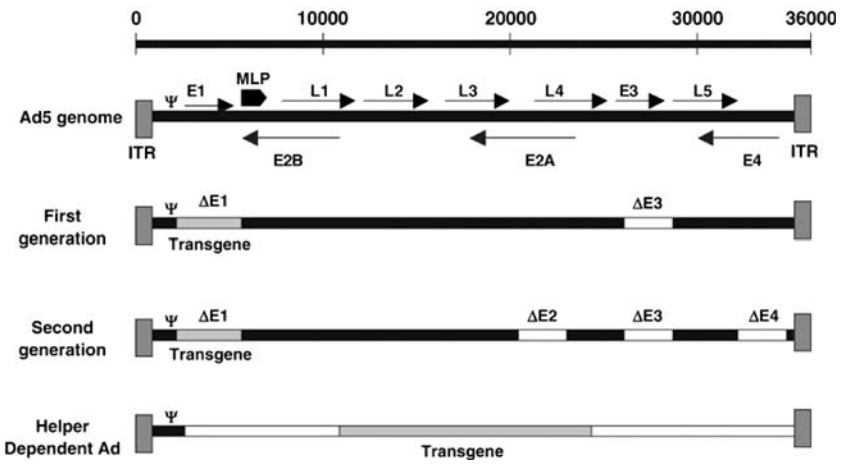

Fig. 1 Schematic map of adenovirus vectors. The genome of adenovirus type 5 is shown schematically and early transcripts are represented by E1-E4, late transcripts by L1-L5 regions, the major late promote by MLP, and the packaging signal by Ψ. Deletions for First Generation, Second Generation, and Helper-dependent vectors are indicated using a "Δ" before the genome region. (Adapted from Alba et al. (2005) *Gene Therapy, 12*, S18–S27.)

36 kb of cDNA; they do not integrate into the cell; and they allow relative easy production of high titer virus.

Finally, the location of CAR receptors tends to focus the vectors into certain tissues such as liver, and this can defeat attempts at targeted gene delivery to extra-hepatic organs. For that reason, the adenovirus fiber proteins have been modified to alter CAR binding. To further improve adenoviral vectors, strategies include altering the receptor-binding via fiber pseudotyping and insertion of heterologous sequences into the fiber nob that permit targeting to different cellular "addresses" and avoidance of anti-viral immune responses [24–27]. Use of bi-specific antibody that binds both to the fiber and to a target cell has also been used to target adenoviral vectors [28, 29]. Finally, coating the vector with polypeptides that can be linked to targeted surface proteins has increased the usefulness of these vectors. For example, the peptide that binds to endothelial cells, SIGYPLP, can be inserted into the adenoviral vector fiber to allow the virus to bind to receptors for this peptide [30, 31]. Ablation of the CAR binding site on the adenoviral fiber can further enhance the specificity of targeting by limiting the binding of virus that increases background effects [32].

An example of the use of adenoviral vectors in a diabetic NOD mouse model has been described by Luo et al. [4] in an attempt to induce immune tolerance and modify the development of diabetes. In this experiment, human tumor growth factor $\beta 1$ (hTGF-$\beta 1$) was introduced into mice by intravenous injection, and this led to increased levels of circulating hTGF-$\beta 1$. Human TGF-$\beta 1$ is known to induce immune tolerance by enhancement of T-regulatory lymphocytes (Treg). The diabetic NOD mice that received the hTGF-$\beta 1$ vector had significantly more periods of normoglycemia than did the control mice that received either an adenoviral vector with no gene or had no treatment at all (Fig. 2). Restoration of immune tolerance also increased the infiltration of CD25+ and FOXP3+ lymphocytes in the pancreas of these mice with resultant increase in the production of insulin [4]. This induction of immune tolerance was associated with an increased production of insulin and transient correction of the hyperglycemia [4].

Molecular engineering of human hepatocytes has been proposed as a method of treating diabetes. Sapir and colleagues have reported the successful hepatocyte to beta cell transdifferentiation using adult human liver cells. In this method, the pancreatic duodenal homeobox gene 1 (PDX-1) was delivered to explanted human hepatocytes using an adenoviral vector [9]. These hepatocytes made insulin in vitro and when injected into diabetic NOD/SCID mice, caused a gradual but significant decrease in the blood glucose levels. This was associated with increasing serum levels of human C-peptide (Fig. 3).

Lentiviral vectors

Oncoretrovirus vectors have been used most widely in gene therapy because of the relatively large capacity for carrying transgenes and because they can integrate and induce stable expression of these genes. In addition, the vectors have relative ease of production in packaging cell lines. The disadvantage of retrovirus vectors is that they undergo random integration into the host genomes, and this can lead to insertional mutagenesis. One human trial resulted in T-cell lymphoma following retrovirus gene therapy [33]. These events severely curtailed the interest in pursuing gene transfer methods using retrovirus vectors.

Lentiviral vectors are derived from HIV-1 and related viruses including feline leukemia virus, equine infectious anemia virus, and foamy virus [34]. Lentivirus can also integrate into the host genome, but the integration tends to occur in gene coding regions whereas retroviral vectors tend to insert into the 5′ control region [35]. Thus, it is anticipated that there will not be the same risk of insertional mutagenesis with use of lentivirus vectors. The HIV-based vector also has the advantages of being able to integrate into non-dividing cells and to induce long-lived gene expression. A disadvantage of lentiviral vectors is that they have not yet been proven to be safe in human trials. Like adenoviral vectors, lentiviral vectors have been deleted of certain genes that are necessary for AIDS pathogenesis. The first generation lentiviral vectors substituted the viral envelope with the envelope of vesicular stomatitis virus glycoprotein (VSV-G) [34, 36]. This allowed the virus to infect multiple types of tissue

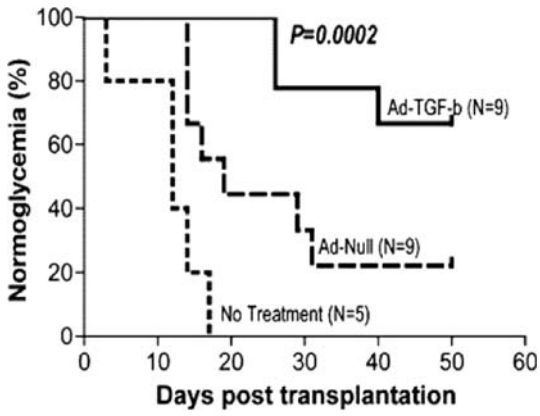

Fig. 2 Restoration of Immune Tolerance Using AdV-TGF-$\beta 1$ in diabetic NOD mice. Female diabetic NOD mice received tail vein injection of Ad-TGF-b, an adenovirus vector encoding transforming growth factor-$\beta 1$ (TGF-$\beta 1$), or control vector Ad-null, or equivalent volume of saline (no treatment). Seven to 14 days later, the mice received syngeneic islet cell transplants. The data are presented as a Kaplan–Meier plot of normal glycemia (non-diabetic state), defined as lack of two consecutive blood glucose levels greater than 250 mg/dL on two separate occasions. (Adapted from Luo et al. (2005) *Transplantation, 79*, 1091–1096)

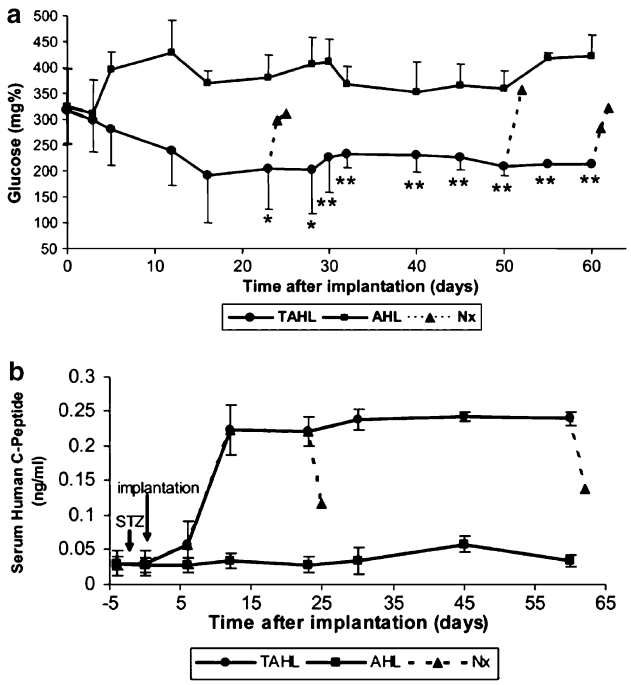

Fig. 3 Transdifferentiated adult human liver (TAHL) cells ameliorate hyperglycemia in NOD-SCID mice. (**a**) Streptozotocin (STZ)-induced diabetic NOD-SCID mice were implanted with TAHL cells transduced with an adenovirus vector encoding pancreatic duct homeobox-1 (pdx-1) gene sequence or with untreated adult human liver (AHL) cells, and the effects on glucose levels (mean mg% ± SE) are shown at the indicated time points after implantation. Dash lines indicate the change in glucose levels after nephrectomy (Nx). (**b**) Serum human C-peptide levels are shown for these same two groups. $*P < 0.05$; $**P < 0.01$. (Adapted from Sapir et al. (2005) *PNAS, 102*, 7964–7969.)

and was no longer tropic only for CD4+ cells. A second generation lentiviral vector resulted from the removal of the accessory genes *vif*, *vpr*, *vpu*, and *nef* [36]. Finally, a third generation lentiviral vector substituted a CMV promoter for the long terminal repeat (LTR) promoter of the virus and deleted *tat* [37]. This vector is termed a self-inactivating vector because once it infects the cell and integrates as a provirus, it has diminished capacity for reactivation of viral infection because of the altered proviral LTR. The difficulty with this vector is that one must supply the helper functions, including Rev function, reverse transcriptase function, and capsid and envelope, necessary for packaging of the vector into non-replication competent lentiviral vector structures that are then capable of infecting cells. Lentiviral vectors have been approved for human use, and several subjects have received T-cells transduced with a lentiviral vector made in a study completed by VirXsys Inc. [38]. An increasing number of lentiviral vector-based treatment protocols are in either development or the approval process at present.

Lentiviral vectors have been used in animal models to induce growth of islet cells ex vivo [10]. This experiment addressed the important problem of how to increase the number of donor islet cells available for transplantation. The lentiviral vector used encoded a chimeric signaling receptor that could then be activated and stimulate cell proliferation using a chemical inducer of dimerization (CID). In this gene-switch system, the CID was a bivalent cell-permeable compound that binds simultaneously to two KFBP12 protein domains [39]. KFBP12 domains were inserted into type 1 fibroblast growth factor receptor (FGFR) or into the erythropoietin receptor (EpoR) and then encoded by a lentiviral vector. Lentiviral vectors have been reported to transduce islets in a stable fashion [40–42], and the vector used for the gene-switch experiments was a pseudo-type vector having the lymphocytic choriomeningitis virus envelope. The chimeric receptors, when expressed in cells, would turn on the gene-switch when activated by treatment with CID. The gene-modified human islets, expressing either the chimeric FGRF or the EpoR, demonstrated increased proliferation when treated with CID. When these modified islets were subsequently transplanted into diabetic SCID mice, the glucose levels were lower using the EpoR gene-switch, and the glucose-lowering effect lasted for approximately 90 days (Fig. 4).

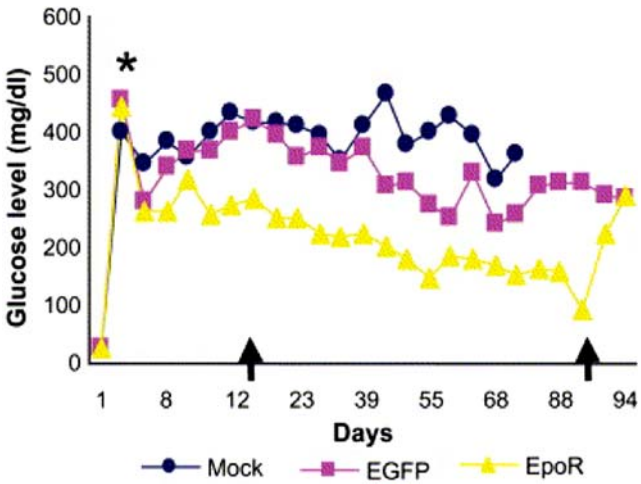

Fig. 4 Glucose levels following transplantation of islets modified for inducible growth response in diabetic SCID mice. SCID mice previously treated with streptozotocin to induce a diabetic state were transplanted with islets transduced or untransduced with JAK/STAT pathway receptors modified to contain a growth signal (GS) responsive to a chemical inducer of dimerization called AP20187. The receptors were cloned into lymphocytic choriomeningitis virus envelope-pseudotyped HIV-based vectors as type I fibroblast growth factor receptor (FgfR) with or without the GS (FgfR-GS) and erythropoietin receptor (EpoR) with or without the GS (Epo-GS). The control islets were transduced with a vector encoding green fluorescent protein. Ten days post-transplantation, mice were treated IP with 2 mg/kg AP20187 (arrow at day 15) and repeated every other day thereafter. The recipient kidney was removed at day 91 (second arrow). (Adapted from Kobinger et al. (2005) *Molecular Therapy, 111*, 105–111.)

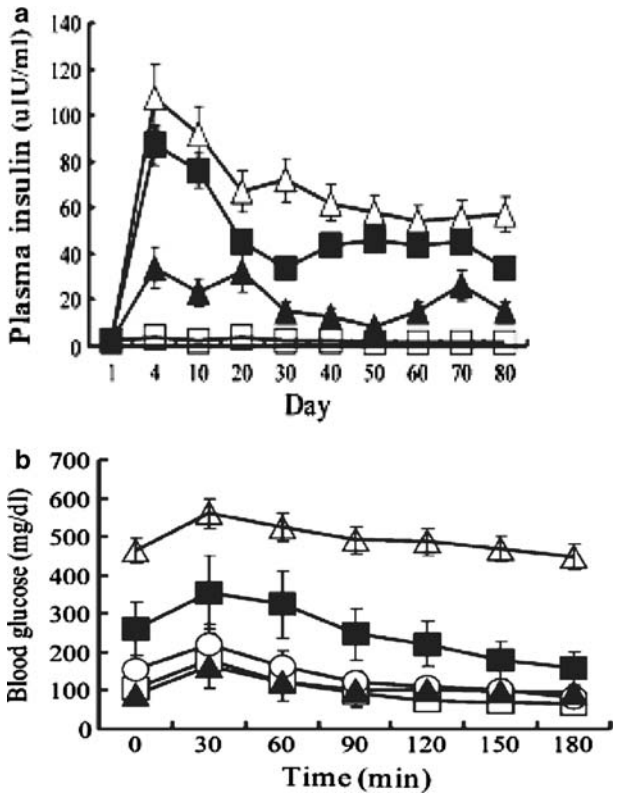

Fig. 5 AAV-induced insulin production in treated diabetic mice and its effect on hyperglycemic control. (**a**) An AAV vector encoding furin-modified human pre-proinsulin (rAAV-hPPI(F12) was injected by portal vein into streptozotocin (STZ)-induced diabetic rats in doses as follows: white triangles, 2×10^{12} units/dose; black squares, 1×10^{12} units/dose; black triangles, 5×10^{11} units/dose; white squares, control vector expressing green fluorescent protein 1×10^{12} units/dose. Plasma insulin levels (mean ± SD) were measured after 6 h fasting. (**b**) Blood glucose levels were measured during intraperitoneal glucose tolerance tests in non-diabetic rats (black triangles), and in STZ-induced diabetic rats after rAAV-hPPI(F12) with vector doses as follows: black squares, 5×10^{11} units/dose; white circles, 1×10^{12} units/dose; white squares 2×10^{12} units/dose; white triangles, control vector 1×10^{12} units/dose). (Adapted from Park et al. (2005) *Journal of Gene Medicine, 7*, 621–629.)

Adeno-associated virus vectors

Adeno-associated virus (AAV) is a small non-enveloped single-stranded DNA virus from the *Parvovirus* family. The advantage of AAV as a genetic vector is that it is non-pathogenic in nature, and it is replication incompetent. The virus replicates in mammals only in the presence of a helper virus such as adenovirus. With helper virus support in vitro, AAV in can be grown to high titers and has been used safely in human gene therapy trials. The disadvantage of this vector is that it can only hold 5-kb of cDNA. Nevertheless, using the appropriate gene sequence, this vector is becoming increasingly useful in gene transfer research. AAV-based vectors have been used in animal models of diabetes and have restored insulin function in several studies. Park et al. were able to transduce cells using a recombinant AAV containing furin-modified human insulin, and after portal vein injection of the vector into the diabetic rats, plasma insulin levels were elevated, and the liver cells showed evidence of insulin production (Fig. 5a). Subsequent glucose tolerance tests in these treated diabetic rats showed significant improvement after introduction of the recombinant AAV vector (Fig. 5b).

Summary

Genetic vectors are just emerging into an era when they will have increasing application to the treatment and prevention of diabetes. To date, there has been only a few gene transfer trials in diabetic patients, and no gene therapy approaches targeting the restoration of insulin levels or the correction of the basic autoimmunity underlying the disease. Yet, preclinical data suggest that several genetic approaches are feasible. If gene transfer could correct any metabolic disease, it would likely be type 1 diabetes because of the multiple approaches available for prevention or treatment. Such strategies would include immune tolerance induction, restoration of insulin response to glucose load, ex vivo expansion of islet cells, protection of expanded islet cells from immune recognition, and rehabilitation of vascular insufficiency and reversal of neuropathy in advanced diabetes. The vectors are now available, and these genetic approaches for prevention and treatment of diabetes by gene transfer vectors are likely to become reality in the future.

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