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RESEARCH ARTICLE

Adenoviral vector-mediated insulin gene transfer in the mouse pancreas corrects streptozotocin-induced hyperglycemia

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Therapy for type 1 diabetes consists of tight blood glucose (BG) control to minimize complications. Current treatment relies on multiple insulin injections or an insulin pump placement, β-cell or whole pancreas transplantation. All approaches have significant limitations and have led to the realization that novel treatment strategies are needed. Pancreatic acinar cells have features that make them a good target for insulin gene transfer. They are not subject to autoimmune attack, a problem with pancreas or islets transplantation, they are avidly transduced by recombinant adenoviral vectors, and capable of exporting a variety of peptides into the portal circulation. Recombinant adenoviral vectors were engineered to express either wild-type or furin-modified human insulin cDNA (AdCMVhInsM). Immunodeficient mice were made diabetic with streptozotocin and injected intrapancreatically with the vectors. BG and blood insulin levels have normalized after administration of AdCMVhInsM. Immunohistochemistry and electron microscopy showed the presence of insulin in acinar cells throughout the pancreas and localization of insulin molecules to acinar cell vesicles. The data clearly establish a relationship between intrapancreatic vector administration, decreased BG and elevated blood insulin levels. The findings support the use of pancreatic acinar cells to express and secrete insulin into the blood stream. Gene Therapy (2001) **8,** 1480–1489.

Keywords: *adenovirus; furin-modified human insulin gene; diabetes; glucose; insulin*

Introduction

Type 1 diabetes is an autoimmune disease that culminates in complete destruction of insulin producing β-cells. The Diabetes Control and Complications Trial has shown that tight control of blood sugar results in fewer complications.[1–3] Existing strategies for the treatment of type 1 diabetes have sought to replace insulin in a physiologic way (multiple injections, insulin pump) or replacement of β-cell mass (islet or whole pancreas transplant).[4–7] Both approaches have limitations, hence new strategies are required for optimal treatment of hyperglycemia and prevention of complications. In particular, the autoimmune process responsible for initial complete destruction of β-cells in the islets of Langerhans continues to plague the use of islet transplantation. Alternative cells are therefore being studied as a source of insulin production.[8–10]

Processing of proinsulin to mature insulin requires two prohormone convertases relatively specific to the β-cell.[11–17] Several studies have shown that it is possible to process proinsulin to its mature form in non-β-cells by engineering furin cleavage sites in the proinsulin molecule.[18–28] When 293 cells are transfected with a human furin modified proinsulin cDNA, efficient processing and constitutive secretion of biologically active insulin was documented.[20] In THP-1 (monocyte-derived) and C2C12 (myoblast-derived) nonendocrine cell lines, transfection with a cDNA plasmid containing furin-modified human proinsulin gene produced mature human insulin.[25] A hepatoma cell line was infected with adenoviral vector expressing human insulin with furin cleavage sites. Cells synthesized both proinsulin and mature insulin, which functionally was identical to that of authentically processed insulin. Normoglycemia was seen *in vivo* when vector was injected into the external jugular vein of streptozotocin-induced diabetic mice.[18] Naked plasmid carrying furin-modified human insulin gene was injected into the skeletal muscle of streptozotocin-induced diabetic mice. Ectopic secretion of mature insulin into the blood caused reversal of hyperglycemia.[26] Streptozotocin-induced diabetic mice were transduced by retrograde ductal injection of the pancreas with a plasmid containing the furin-modified human insulin. Treatment with this plasmid resulted in reduction of blood glucose levels to normal ranges.[29] Mice transgenic for a GIP/insulin construct responded to glucose with physiologic insulin release. The cell type was believed to be the gut-derived K cell.[30]

The pancreatic acinar cell is a good target for ectopic expression of insulin. Acinar cells are capable of

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exporting protein into the bloodstream, as well as the ductal lumen, and blood borne proteins enter the portal circulation.[29,31,32] High level gene transfer in the rat and mouse pancreas have been shown using adenoviral vectors. Intra-ductal and direct intra-pancreatic injections are efficient ways to deliver adenoviral vector into the mouse pancreas.[33–38] Advantages of using adenoviral vector include high level expression, wide tissue tropism, ability to achieve high titers, a short period between transduction and expression (2–4 days), and a large insert capacity. Sophisticated techniques allow subcellular localization of transgene product.[39,40] These preliminary data led to a hypothesis that intra-pancreatic administration of recombinant adenoviral vector expressing the furin-modified human insulin gene might be capable of reversing diabetes-associated hyperglycemia.

Results

Correction of high blood glucose level after injection of the vector expressing furin-modified human insulin cDNA

Serial BG levels in CD-1 nude immunodeficient mice are represented in Figure 1a. At day 0 (before the intra-peritoneal injection of streptozotocin) mice had normal BG values. On day 7 after streptozotocin administration, mice which became diabetic received intra-pancreatic injection of adenoviral vector. In the group that received furin-modified human insulin cDNA (AdCMVhInsM) 37% of the mice became normoglycemic (BG level below 190 mg/dl) at day 9 (day 2 after vector administration, which corresponded to the beginning of transgene expression) compared with controls and the number of mice with normal BG increased to 71% by day 37. Corresponding to normalization of blood glucose levels, vector-treated mice were also noted to have elevations in mean plasma insulin levels (Figure 2a, b). In the group that received wild-type human insulin cDNA (AdCMVhInsWT) and the control group, no mice became normoglycemic.

In CD-1 nude mice, BG levels were more variable. Some mice experienced persistent normoglycemia, but other mice had intermittent periods of hyperglycemia followed by the periods of normoglycemia. The variability in the response of the nude mice to the vector suggested an immune mechanism. The next experiments were performed in RAG-1-immunodeficient mice to assess both strain variability and persistence. Two different doses of the adenoviral vector expressing furin-modified human insulin gene, were injected in the RAG-1 mice (6×10^{10} and 1×10^{11} pt/mouse). RAG-1 mice, which received a low dose of furin-modified human insulin vector (6×10^{10} pt/mouse), had normal or slightly higher than normal BG levels. One mouse reverted to being hyperglycemic at day 21. All RAG-1 mice, which received the high dose of furin-modified human insulin vector (1×10^{11} pt/mouse), were persistently normoglycemic (BG level below 190 mg/dl) to the end of the experiment. There was a trend to more significant lowering of BG levels in the group that the received higher dose of furin-modified insulin vector (Figure 1b).

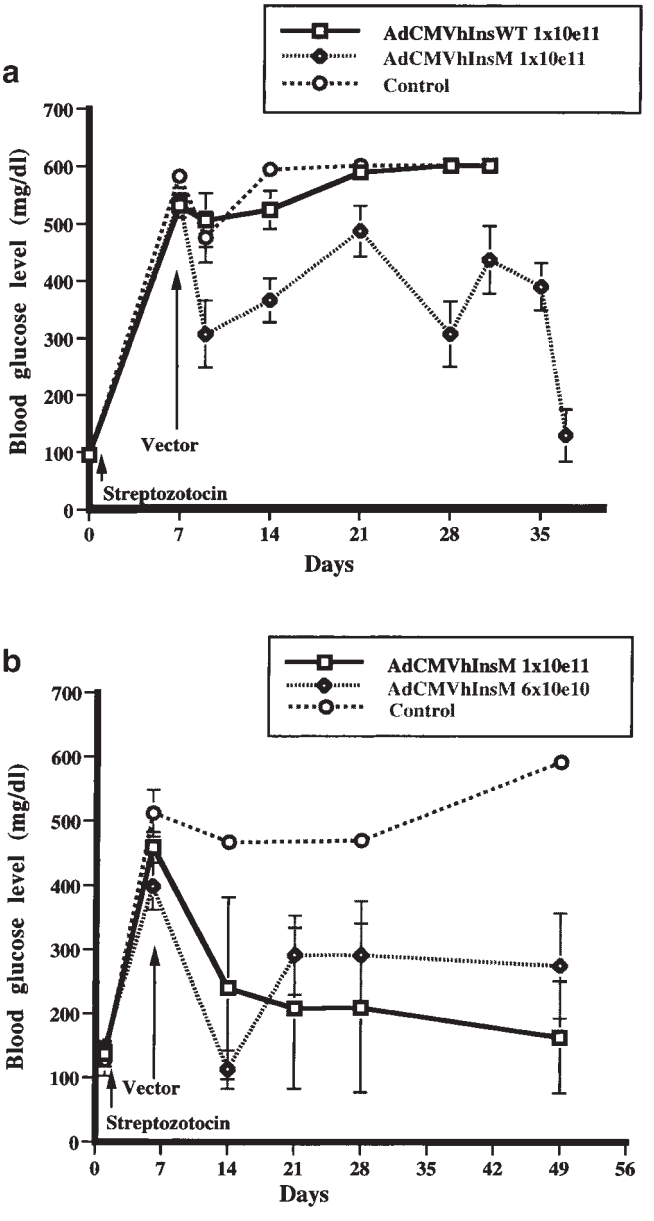

Figure 1 *Blood glucose levels in streptozotocin-treated diabetic mice injected with different doses of AdCMVhInsWT and AdCMVhInsM vectors. (a) CD-1 nude and (b) RAG-1 immunodeficient mice (error bars represent mean ± s.d.).*

Correction of ketoacidosis and glucose tolerance after injection of the vector expressing furin-modified human insulin cDNA

Separate experiments in CD-1 nude mice were performed to determine blood ketone levels and glucose tolerance. Control streptozotocin-induced diabetic mice and vector-treated experimental mice (wich received AdCMVhIns M) were selected for this experiment. Serial blood ketone levels in CD-1 nude immunodeficient mice are represented in Figure 3a. At day 0 (before the intra-peritoneal injection of streptozotocin) mice had normal blood ketone levels. Mice, which became diabetic on day 4 after streptozotocin administration, also developed ketoacidosis (blood ketone levels doubled compared with day 0), and these mice received intra-pancreatic injection of

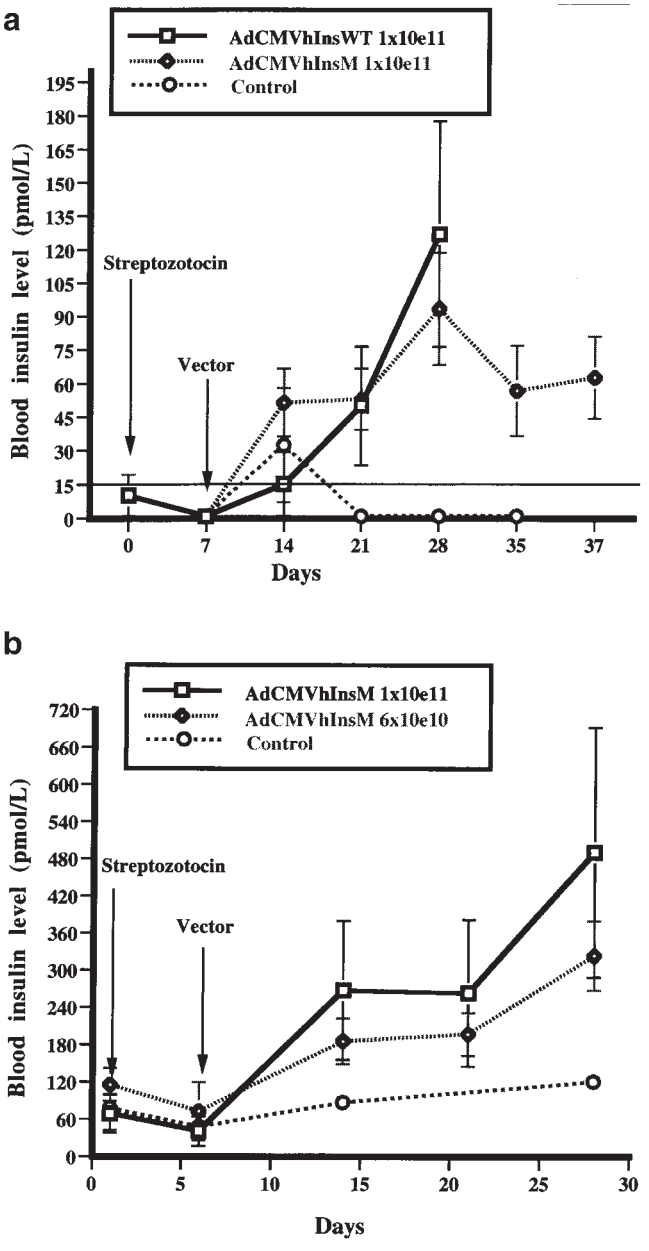

Figure 2 *Blood insulin levels in streptozotocin-treated diabetic mice injected with different doses of AdCMVhInsWT and AdCMVhInsM vectors. (a) CD-1 nude and (b) RAG-1 mice (error bars represent mean ± s.d.).*

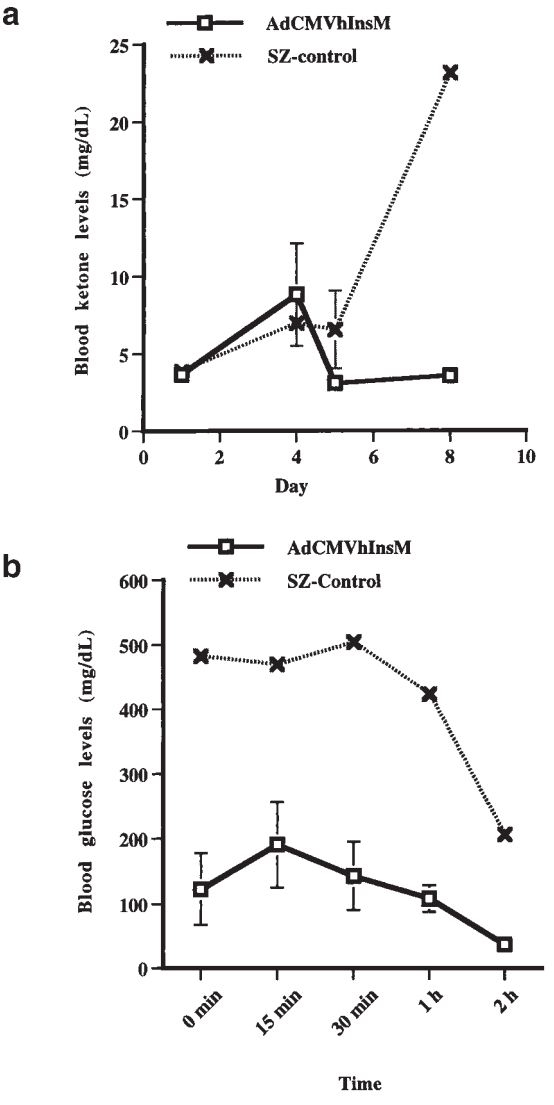

Figure 3 *(a) Blood ketone levels (CD-1 nude mice). Streptozotocin was administered at day 1. At day 4 blood ketone levels were raised in both groups. AdCMVhInsM was administered to the experimental group at day 4. At day 8 control mice developed severe ketoacidosis, but in the experimental group blood ketone levels became normal. (b) Glucose tolerance tests in CD-1 nude mice at day 8: controls and vector-administered. The experimental mice developed glucose tolerance in response to intraperitoneal administration of 0.5 mg per gbw of glucose solution.*

adenoviral vector. On the following day, blood ketone levels decreased to normal values in mice which received vector, but not in streptozotocin-treated control mice. At day 8 after administration of streptozotocin (4 days after vector administration to the experimental group) experimental mice continued to have normal blood kenone levels, but control mice developed very high level of ketones in the blood and all of them were killed.

Glucose tolerance tests are represented in Figure 3b. In response to intra-peritoneal injection of 0.5 mg/gbw (gram body weight) of glucose solution, the vector-treated experimental group developed high normal values of blood glucose within 15 min of glucose administration. Blood glucose levels returned to normal by 2 h. Streptozotocin-treated control mice did not respond.

Blood insulin levels increase after injection of the vector expressing furin-modified human insulin cDNA

RIA was performed to detect the presence of human insulin in mouse serum. The assay had very little cross-reactivity with mouse insulin, represented by values below detectable level (15 pmol/l). The average normal random level of mouse insulin in the serum of CD-1 nude and RAG-1 mice, detected with ELISA before injection of streptozotocin, was 44.8 ± 5.7 pmol/l and 56.7 ± 14.6 pmol/l, respectively.

Two days after vector administration detectable transgene expression was seen. In the group that received furin-modified human insulin vector, blood insulin level was significantly higher than in control animals (Figure 2a, b). High blood insulin value was strongly correlated with appropriate low BG level in the same mouse. Blood

insulin levels were higher in the group that received higher vector dose (1×10^{11} pt/mouse) (Figure 2b). There was some elevation of blood insulin levels in mice that received AdCMVhInsWT vector (Figure 2a), suggesting that exocrine pancreas can process wild-type human proinsulin molecule to a certain extent as well.

Survival of mice receiving furin-modified human insulin vector

Survival was improved, with more animals surviving in the group that received AdCMVhInsM vector. After injection of streptozotocin, mice became severely hyperglycemic, they developed polyuria, polydipsia, became moribund with progression to dehydration and required euthanasia. Figure 4a and b represents mortality rates in CD-1 nude mice and RAG-1 mice. The mortality rate was significantly lower in the group which received furin-modified human insulin gene. Almost all control animals and those which received wild-type human insulin vector, were dead by day 35 in nude mice, and only one mouse per group survived in the RAG-1 experiment. In both groups (RAG-1 and CD-1 nude mice) all of the animals which received AdCMVhInsM vector and survived the first 48 h after vector administration (when transgene expression started) were alive until the end of the experiment (except mice killed at different time-points to harvest the pancreas for immunohistochemistry). We decided to terminate the experiment in CD-1 nude mice at day 37 and in RAG-1 mice at day 49, because all mice in the control group and the group that received AdCMVhInsWT, died. Previous work of our laboratory have shown that length of transgene expression in nude and RAG immunodeficient mice lasts up to day 60 or longer.[36]

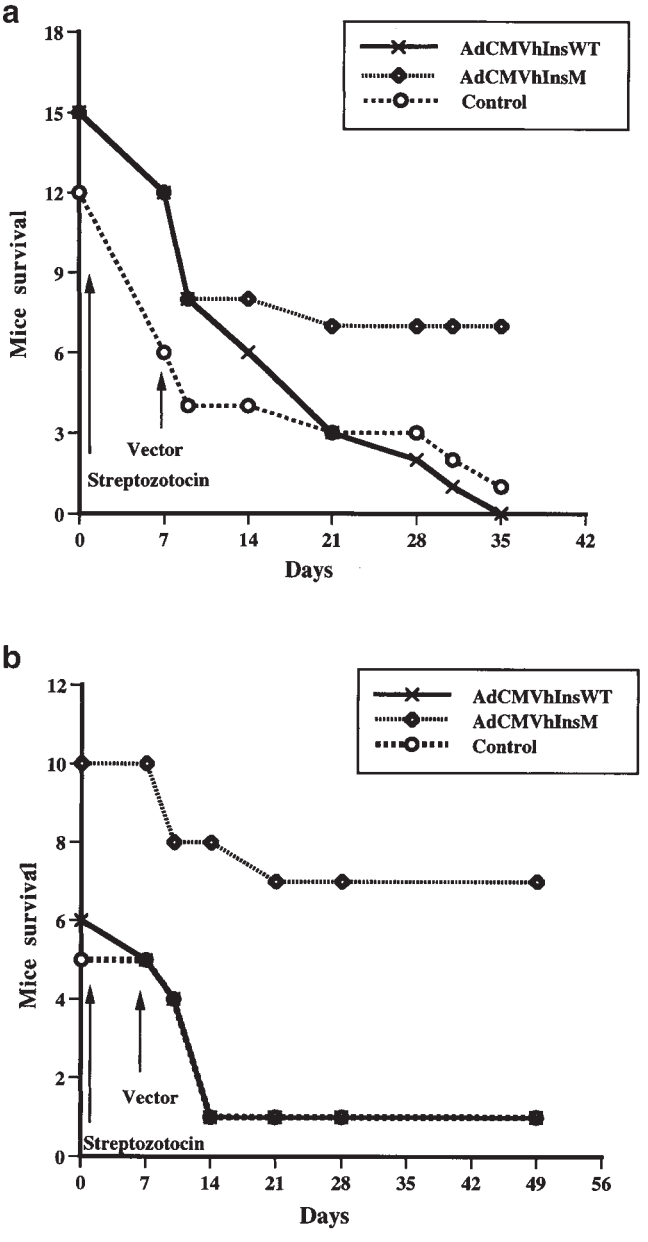

Figure 4 *Mortality rate in diabetic (a) CD-1 nude and (b) RAG-1 mice after streptozotocin administration, followed by treatment with insulin expressing vectors.*

Insulin immunohistochemistry of vector-expressing acinar cells

To demonstrate insulin transgene expression and to determine the cell type that expressed insulin, immunohistochemical staining for insulin using anti-human insulin antibody was performed. Mice injected with streptozotocin have markedly decreased or complete absence of DAB (brown) staining in the islets of Langerhans and no brown staining in the acinar tissue, consistent with depletion of β-cells (Figure 5a and b). Figure 5c represents day 7 after intrapancreatic injection of AdCMVhInsM vector in streptozotocin-treated diabetic mice. Pancreas injected with this vector shows the presence of positive staining for insulin in acinar cells throughout the pancreas. Presence of positively stained acinar cells in the pancreas injected with AdCMVhInsWT was also seen, despite the inability of this vector to normalize blood sugar. As noted in Figure 5b and c, there is very little inflammation at the site of injection.

Immunoelectron microscopic detection of human insulin in mouse pancreatic acinar cells

To document the sub-cellular localization of insulin in transduced acinar cells, immunoelectron microscopy was performed. Normal β-cells show the presence of normal granules labeled with gold-labeled anti-human insulin antibody (Figure 6a). Normal acinar cells are negative for gold particles consistent with absence of insulin (Figure 6b). In contrast, secretory vesicles in the acinar cells transduced with furin-modified insulin expressing vector are positive for gold particles (Figure 6c). In the positive cells, label is clearly located in secretory vesicles close to the apical region and baso-lateral membrane. The ratio of apical to baso-lateral granules is approximately 2:1. Positively labeled acini were also noted very close to the capillaries. There is very little labeling in the ER lumen. Other cells, including fibroblasts and vascular endothelial cells, were negative.

Discussion

The work presented here extends work done previously and shows the ability of recombinant adenoviral vectors to efficiently transduce pancreatic acinar tissue with a functional insulin gene.[33–38] Non-β-cells are being considered for the ectopic expression of insulin due to con-

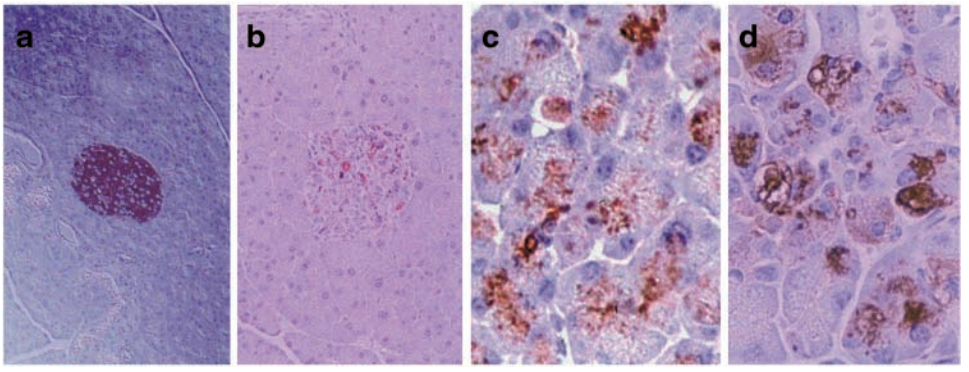

Figure 5 *Insulin immunohistochemical staining of mouse pancreas. (a) Normal islet of Langerhans. Brown staining (DAB) for insulin; (b) Islet of Langerhans in the mouse treated with intra-peritoneal injection of streptozotocin. Note that the islet has essentially no positive staining for insulin. Note also the absence of positive staining in the pancreatic acinar tissue; (c) Pancreas injected with vector carrying furin-modified human insulin gene and stained with monoclonal anti-human insulin antibody (×100) at day 7 after injection. Pancreas injected with this vector shows the presence of positive staining for insulin (brown staining, DAB) in acinar cells throughout the pancreas (d) Pancreas injected with vector carrying wild-type human insulin gene and stained with monoclonal anti-human insulin antibody (×100). Note the same distribution of the positive staining for insulin throughout the acinar tissue.*

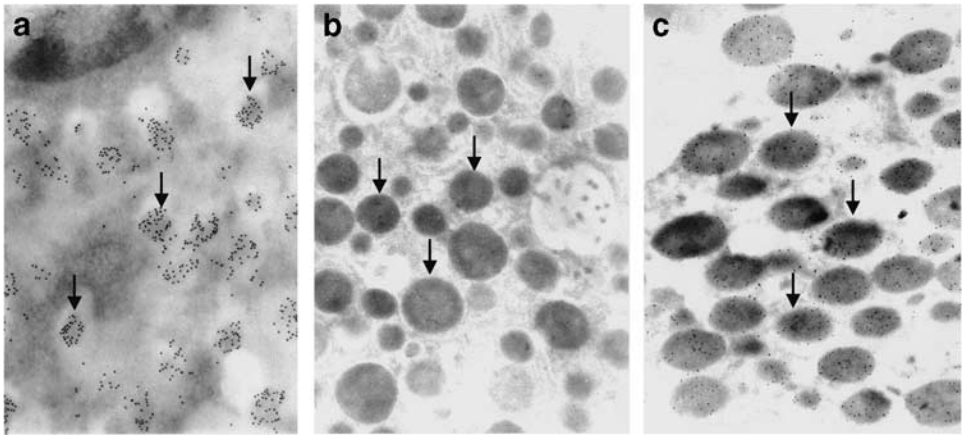

Figure 6 *(a) Immunoelectron microphotogram of a normal beta cell. Arrows show beta cell granules with insulin (dark dots represent gold particle conjugated with anti-human insulin antibody). (b) Immunoelectron microphotogram of a normal acinar cell. Arrows point at the normal secretory vesicles – no insulin staining in the vesicles is seen. (c) Immunoelectron microphotogram of an acinar cell expressing furin-modified insulin gene. Arrows represent positive insulin staining within acinar secretory vesicles.*

cerns about autoimmune destruction of transplanted islets of Langerhans. Type I diabetes results from a genetically susceptible, immune-mediated, selective destruction of more then 90% of insulin-secreting β-cells. Pancreatic islets exhibit insulitis, which is characterized by an infiltration of T-lymphocytes, B-lymphocytes and macrophages. Cell-mediated immune mechanisms are believed to play the major role in β-cell destruction. Antibodies present in the serum include islet cell cytoplasmic antibodies and islet cell surface antibodies (islet cell autoantibodies (ICAs), autoantibodies to insulin (IAAs), autoantibodies to glutamic acid decarboxylase (GAD65), and autoantibodies to the tyrosine phosphatases IA-2 and IA-2). There are also strong HLA associations.[2,41–46]

Use of non-β-cells can circumvent the autoimmune destruction of insulin-containing cells. Viral and non-viral vectors have been used for insulin gene delivery in the different tissues including muscle,[8,26,47,48] liver (hepatocytes),[18,25,27–29,47,49–52] pituitary,[8,53] submandibular gland[29] and exocrine pancreas.[29] The most productive results have been achieved by using vector carrying furin-modified insulin.[18,20,22–27] The studies reported here have demonstrated that injection of adenoviral vectors carrying furin-modified human insulin gene into the pancreatic parenchyma reverses hyperglycemia in streptozotocin-induced diabetic mice. The proinsulin translated from mRNA is processed, correctly localized in secretory vesicles, and prolongs survival. BG levels in all mice were normal before injection of streptozotocin, and markedly elevated after injection. All mice became severely hyperglycemic; they developed polyuria, polydipsia with progression to dehydration and many were killed. Histochemically, mice that received streptozotocin showed markedly diminished positive staining for mouse insulin in the islets of Langerhans. Injection of AdCMVhInsM not only normalized BG levels but also improved the physical condition of diabetic mice. Two days after the vector injection mice started to look healthier, became less dehydrated and had diminished polyuria. The data (detection of human insulin with RIA of mouse serum) indicate that insulin produced by the acinar cells was secreted into the blood stream and was capable of lower-

ing BG levels. Our data, as well as work by Kolodka *et al*,[28] have shown that secretion of insulin not only restored BG levels to normal, or close to normal but also prevented development of ketoacidosis.[47,54] CD-1 nude mice, which received AdCMVhInsM vector and were challenged with intraperitoneal injection of glucose solution, developed glucose tolerance compared with streptozotocin-treated diabetic control mice. This was confirmed by prolonged survival of these animals compared with the animals that received AdCMVhInsWT or controls.

Higher levels of blood insulin were detected in the group that received the AdCMVhInsM vector than in the group which received AdCMVhInsWT vector. Insulin was detectable within 2 days after vector administration in experimental groups, but not the control group, indicating that the development of euglycemia was a result of transgene expression and not recovery of endogenous islets. Immunohistochemical staining of the pancreas showed the presence of positive insulin staining in the cytoplasm of the acinar cells throughout the pancreas. Insulin staining was present in both groups that received AdCMVhInsM or AdCMVhInsWT vectors in qualitatively similar distribution. The fact that high levels of staining in the tissues occurred in both groups, despite the inability of the AdCMVhInsWT to lower blood sugar, is evidence for the ability of the furin-modified cDNA to be converted to biologically active insulin.

The exocrine pancreas is well situated to act as a surrogate for intraportal insulin delivery. Intrapancreatic injection (when the needle was inserted directly into the pancreas) and mobilization of the pancreas resulted in the development of local intrapancreatic inflammation.[38] Direct glandular injection is associated with attraction of inflammatory cells into the pancreas, development of local fibrotic changes, and development of adhesions. These changes in the acinar tissue could potentially result in impairment of blood supply to the insulin-producing part of the pancreas. In larger species, including humans, direct ductal injection could be accomplished without the need for laparotomy.[32,34]

Pancreatic acinar cells have an extensive arteriolar blood supply. The insulo-acinar portal system in the rodent pancreas consists of three types of arterioles according to their destination: the arteriole which supplied the capillary glomerulus of the islet, the arterioles which directly branched out into capillaries around the acini, and the arterioles which supplied the duct system. There are three types of efferent vessels: the insulo-acinar portal vessels that radiated from the islet to join the capillary network in the exocrine pancreas, the emissary venules of the islet, leading directly into the systemic circulation, and the insulo-ductal portal vessels which drained into the peri-ductal capillary network.[55] Pancreatic acinar cells have a well-developed secretory pathway that allows storage and secretion of proteins into the portal circulation.

We report here for the first time the presence of insulin inside the secretory vesicles in the acinar cell. This insulin was processed and shown to traffic to apical, as well as to the baso-lateral membranes. The relative proportion of insulin secreted into the portal circulation *versus* into the lumen of the pancreatic duct is a matter of active investigation. In addition, the consequences of intraportal delivery of high concentrations of biologically active insulin are also under study. Given the avidity with which the hepatocyte can take up and process insulin, portal concentrations of insulin are a matter of some importance. One such effect of hyperinsulinemia is the development of atherosclerosis in rats subjected to heterotopic or orthotopic pancreas transplantation. The study has shown that portal delivery of insulin from the transplanted pancreas is relatively preventive for the atherosclerotic process (plasma cholesterol and triglyceride concentrations were significantly lower) as compared with systemic delivery of insulin.[56] Further, ketosis might be prevented even in the absence of normalization of blood sugar.

Recombinant adenoviral vectors are useful tools for intra-pancreatic gene delivery. They are well-characterized, relatively easy to produce, and have efficient transgene expression and serve well for proof-of-concept experiments. Immune responses to adenoviral vector with host injury and short duration of expression remain major obstacles.[35,36,57,58] AAV vectors are less immunogenic, have prolonged transgene expression (probably secondary to integration) and do not activate cellular or humoral immunity to transgene product.[10,59] Ultimately, vectors with the advantages of both vector systems are desirable.

Another problem in gene therapy specifically for diabetes is physiologic regulation of insulin gene transcription and glucose sensitive secretion.[10,60–62] Regulated gene expression with transcriptional activation is possible. In these experiments a tetracycline-inducible promoter was inserted above the furin-modified insulin coding sequence. An adenoviral vector containing the inducible construct was injected via tail vein and predominantly targeted hepatocytes. Upon stimulation with tetracycline transcription turned on and the mature form of insulin was secreted in the blood stream.[52] Other laboratories have shown the possibility of using an alternative glucose-regulated insulin release and glucose-regulated insulin gene transcription. Glucose-regulated gene transcription is possible by using phosphoenolpyruvate carboxykinase (PEPCK) promoter or the L-type pyruvate kinase (L-PK) promoter inserted above insulin gene sequence. The PEPCK promoter is stimulated by glucagon and inhibited by insulin,[8,23,47,63,64] while the L-PK promoter is triggered by glucose and fructose and inhibited by glucagon.[10,23,24,27,47,65] Glucose-stimulated insulin release was studied using glucokinase and GLUT2 promoters. Glucokinase promoter is stimulated by insulin and inhibited by glucagon.[6,23,47,54,66,68] GLUT2 has a glucose-sensitive effect on insulin secretion but not in the physiological range.[4,23,47,54,66,67] Pancreatic acinar cells have single-channel currents from the baso-lateral surface of pancreatic acini. These channels have Ca^{2+}-mediated neural and hormonal control of pancreatic acinar membrane conductance, which is responsible for the Ca^{2+}-dependent acinar fluid secretion.[69] Some data indicate that administration of calcium inophore A23187 can stimulate amylase release from acinar cells.[70] Another laboratory studied a role of Rab3D in regulated amylase secretion from pancreatic acini.[71] Rab3D, a member of the ras-related GTP-binding protein Rab family, localizes to secretory granules of various exocrine tissues, including acinar cells of the pancreas. Overexpression of Rab3D enhances secretagogue-stimulated amylase secretion through both calcium and GTP pathways. Using of

recombinant kinesin, which has recently been localized to zymogen granules of pancreatic acini, also enhanced calcium-stimulated amylase release from pancreatic acini. Kinesin plays a stimulatory role in regulated exocytosis of pancreatic acini and is involved in stimulus-secretion coupling through a cAMP-dependent pathway.[71]

In summary, intrapancreatic administration of insulin-expressing adenoviral vectors can restore euglycemia in a diabetic mouse model. Processing of proinsulin to its active form is possible in pancreatic acinar cells. When compared with cDNA containing a wild-type human insulin, a furin-modified insulin cDNA resulted in a higher response rate, with low blood glucose and high blood insulin levels. The data support the hypothesis that pancreatic acinar cells might be good surrogate cells to express and secrete mature insulin into the portal circulation. The data clearly establish a relationship between intra-pancreatic vector administration, decreased blood glucose and elevated blood insulin levels in a dose-dependent manner. A better effect was achieved with a higher dose of vector (1×10^{11} pt/mouse). Injection of furin-modified insulin prevented development of ketoacidosis, improved glucose tolerance and decreased the mortality rate in streptozotocin-treated diabetic mice. Future goals include the development of vectors with less immunogenicity for prolonged transgene expression, longer duration of expression, and tightly regulated insulin secretion.

Materials and methods

Recombinant adenoviral vectors

Recombinant E1-deleted human adenoviral vector containing a high-level constitutive cytomegalovirus promoter-enhancer was engineered to contain the human insulin gene wild-type AdCMVhInsWT and human insulin gene-modified with furin cleavage sites – AdCMVhInsM.[11,12,14,16,19–22] The cDNA for the insulin gene is modified to allow proteolysis and processing of transgene product to mature insulin in non-beta cells by the ubiquitous endopeptidase, furin. Vector was produced by homologous recombination in the 293 human embryonic kidney cell line. Two different vector doses of AdCMVhInsM and AdCMVhInsWT were used in this study: 6×10^{10} and 1×10^{11} particles/mouse (pt/mouse).

Animals and methods

Immunodeficient CD-1 nude and RAG-1 mice, 4–8 weeks old, were purchased from Taconic, Germantown, NY, USA. Mice were made diabetic with streptozotocin injected intraperitoneally in a single dose of 200 mg/kg. Depletion of β-cells was confirmed with immunohistochemical staining of the pancreas, as well as profound hyperglycemia. Mice that became hyperglycemic were randomized to experimental groups, which received 6×10^{10} or 1×10^{11} pt/mouse of AdCMVhInsWT and AdCMVhInsM vectors and diabetic controls (no vector). Seven days after streptozotocin administration, mice received direct intra-pancreatic injection of the vector.

Blood was collected by the retro-orbital approach at day 0 (before intraperitoneal injection of streptozotocin), day 7 (before injection of recombinant adenoviral vectors), day 9 (2 days after vector injection) and weekly thereafter. A subset of mice with low BG level in the experimental groups were killed at day 7 after vector administration along with control animals with high BG level. Pancreas was harvested and embedded in paraffin for immunohistochemical staining. Experiments were terminated when all animals in the control group were killed, which corresponded to day 37 in CD-1 nude mice and day 49 in RAG-1 mice.

Vector injection procedure

Mice were anesthetized by intraperitoneal injection of ketamine (70 mg/kg) and xylazine (10 mg/kg). Following laparotomy, the distal pancreas was identified and mobilized. Using a 27-gauge needle, the recombinant adenoviral suspension in a total volume of 100 μl, was directly injected at the several sites of the distal pancreatic parenchyma (two to three sites per pancreas depending on the size of the pancreas). This technique was standardized based upon our preliminary experiments with AdCMVlacZ vector to get expression in maximum of the pancreatic tissue. Vectors were injected in doses of 6×10^{10} pt/mouse (2×10^{10} pt/mouse for each injection if we did three intra-pancreatic injections or 3×10^{10} pt/mouse for each injection if we did two injections) or 1×10^{11} pt/mouse (3.3×10^{10} pt/mouse for each injection if we did three intra-pancreatic injections or 5×10^{10} pt/mouse for each injection if we did two injections), depending on experiment. Abdominal incision was closed with two layers of 4–0 Vicryl (Ethicon, Somerville, NJ, USA). The Institutional Animal Care and Use (IACUC) and Institutional Biosafety Committees of the University of Pennsylvania approved animal procedures and use of recombinant DNA.

Blood glucose measurement and glucose tolerance test and blood ketones measurement

Serial blood glucose (BG) levels were monitored using the Accu-Chek Easy glucometer (Roche Diagnostic, Indianapolis, IN, USA) and Accu-Chek Easy test strips (Roche Diagnostic) with appropriate calibration. BG levels were determined on day 0, before intraperitoneal injection of streptozotocin, day 7 – before injection of recombinant adenoviral vectors and every 2–7 days thereafter. The highest normal BG levels in CD-1 nude and RAG-1-immunodeficient mice, which we measured before injection of streptozotocin, were 180–190 mg/dl. As there is no universal determination of normoglycemia in these mice strains, we defined normal BG as <190 mg/dl based on our data.

Glucose tolerance testing was performed in a separate experiment on CD-1 nude mice. At day 8 (day 4 after the vector administration) experimental mice (which received intra-pancreatic injection of AdCMVhInsM in a dose of 1×10^{11} pt/mouse) and streptozotocin-induced diabetic control CD-1 nude mice were fasted overnight, or at least for 6 h, with free access to water. Glucose was injected intraperitoneally at a dose of 0.5 mg per g of body weight at 1 ml per mouse. Mice were bled by retro-orbital bleeding. Blood glucose measurement was performed before and after the glucose injection at 15, 30, 60 and 120 min using the Accu-Chek Easy glucometer (Roche Diagnostic, Indianapolis, IN, USA).

Serial blood ketone levels were monitored using the Bioscanner 2000 (Polymer Technology Systems, Indianapolis, IN, USA) and Bioscanner ketone test strips 2000

(Polymer Technology Systems) with appropriate calibration.

RIA for human insulin and ELISA for mouse insulin

Serial insulin levels (at times listed above) were determined with a radioimmunoassay (RIA) for human insulin. The RIA for human insulin was performed as described in the manufacturer's protocol for human insulin RIA (LINCO Research, St Charles, MO, USA). 1014 Guinea pig anti-human insulin-specific antibody and lyophilized ^{125}I-human insulin label, HPLC-purified (specific activity 367 μCi/μg) were used in this assay. 1014 Guinea pig anti-human insulin-specific antibody has a 100% specificity in the recognition of mature human insulin and not proinsulin (<0.2%). For each mouse, 25 μl of serum was diluted in 75 μl of insulin buffer (per RIA protocol). Data were expressed in pmol/l. Values below detectable level (15 pmol/l), but which were high then dropped to 0 were calculated as 1 pmol/l.

Baseline mouse insulin levels were determined in CD-1 nude and RAG-1-immunodeficient mice with ELISA for mouse insulin. The ELISA for mouse insulin was performed with ultrasensetive rat anti-mouse insulin antibody (American Laboratory Product Company, Windham, NH, USA) as described in the manufacturer's protocol for ultrasensitive rat insulin EIA (American Laboratory Product Company). For each mouse, 5 μl of serum was used. Data were expressed in pmol/l.

Immunohistochemistry

Pancreatic tissue was harvested, fixed in 10% neutral buffered formalin, and embedded in paraffin. Tissue sections (5 μm) were cut with a cryotome and placed on Superfrost/Plus microscope slides (Fisher Scientific). Sections were deparaffinized and rehydrated through a graded series of alcohol to distilled H_2O according to standard procedures. Endogenous peroxidase was blocked in a solution of 3% H_2O_2 in MeOH for 30 min at room temperature. Non-specific staining was blocked with Superblock Blocking Buffer in PBS (Pierce, Rockford, IL, USA) for 30 min at room temperature. Using the NEXES automated immunohistochemistry stainer (NEXES IHC staining system, Ventana Medical Systems, Inc., Tucson, AZ, USA), slides were incubated with monoclonal mouse Lyoph-insulin antibodies (Clone 2D11-H5) (Ventana Medical Systems) for 32 min at 37°C, and a secondary antibody of biotinylated goat anti-mouse IgG (Vector Laboratories, Inc., Burlingame, CA, USA) diluted 1:500 in 1 × PBS for 10 min at 37°C. Detection was done using the Ventana Basic DAB Detection Kit (Ventana Medical Systems). Slides were incubated in avidin HRPO for 8 min, and then DAB+ H_2O_2 substrate for 8 min followed by a light hematoxylin/bluing reagent counterstain.

Immunoelectron microscopy of the mouse pancreatic acinar cells

The mouse specimens were processed following previously reported methods[39,40] with some modifications. Briefly, all the pancreatic tissue were surgically collected fresh from the animal, trimmed into 2-mm pieces and immediately fixed with 2% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M PBS for 1 h. The samples were then treated with 10 mM NH_4Cl for 10 min, washed with cold PBS, dehydrated at –20°C with ethanol, embedded in Lowicryl K4M medium (Ladd, Burlington, VT, USA), and polymerized at –20°C with UV light (365 nm wavelength) for 5 days. Ultrathin sections (~90 nm) were cut with a diamond knife and mounted on Formvar-coated nickel grids. The sections were treated for 40 min with a blocking buffer containing 1% BSA, and 2% normal goat serum, and incubated for 3 h with a monoclonal antibody raised against human insulin (Ventana Medical Systems). Negative control sections received identical treatment with the only omission being primary antibody. To ensure specificity, serial sections from the same tissue block were also labeled simultaneously. Sections were incubated for 30 min with goat-anti-mouse antibody conjugated with 10 nm colloidal gold. The sections were washed several times and fixed with 2% glutaraldehyde for 5 min, briefly stained with uranyl acetate and lead citrate, and examined with a Philips CM-100 transmission electron microscope.

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