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Research Paper

Glucose- and Metabolically Regulated Hepatic Insulin Gene Therapy for Diabetes

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Purpose. The purpose of this study was to examine glucose- and metabolically modulation of insulin secretion by rAAV-mediated gene delivery *in vitro* and *in vivo.*
Materials and methods. A recombinant adeno-associated virus vector (rAAV) containing a furin-mutated human insulin gene, driven by the rat insulin I promoter, was used in this study. Glucose-responsive secretion of human insulin was determined by treating rAAV-transduced Huh7 human hepatoma cells with varying concentrations of glucose, with or without insulin secretagogues. Glucose- and metabolically modulated secretion of human insulin in the streptozotocin (STZ)-induced diabetic mice was assessed by intrahepatic administration of rAAV-polyethylenimine (PEI) complexes, followed by intraperitoneal glucose tolerance test (IPGTT), with or without theophylline.
Results. Glucose- and metabolically controlled human insulin secretion was obtained in the rAAV-transduced Huh7 cells. Treatment of STZ-induced diabetic animals with rAAV–polyethylenimine (rAAV-PEI) complexes resulted in production of human insulin and amelioration of hyperglycemia. Co-administration of glucose and theophylline in these animals augmented the secretion of human insulin, demonstrating metabolic modulation of insulin secretion *in vivo*. Immunohistochemical examination of the liver sections of rAAV-treated mice confirmed the production of human insulin.
Conclusions. Glucose- and metabolically controlled hepatic insulin gene therapy was obtained both *in vitro* and *in vivo.*

KEY WORDS: diabetes mellitus; insulin; polyethylenimine; recombinant adeno-associated virus; regulated gene therapy.

INTRODUCTION

Insulin-dependent diabetes mellitus (IDDM), known as type 1 diabetes, is caused by autoimmune destruction of insulin- producing pancreatic β-cells. Patients with IDDM often rely on constant administration of insulin, however, large fluctuations in blood concentrations are often observed with insulin therapy. In an effort to mimic the physiological conditions of insulin secretion, attempts have been made by various means to control insulin secretion in response to blood glucose levels. Strategies used for transcriptional control of insulin secretion include the utilization of glucose-responsive elements (GREs) of the rat L-pyruvate kinase (L-PK) gene, inserted into the insulin-sensitive element of rat insulin-like growth factor binding protein-1 (IGFBP-1) promoter (1,2); employment of genetically engineered cells to over-express key regulatory genes such as glucokinase (GK) in skeletal muscle (3,4); the use of phosphoenolpyruvate carboxykinase (PEPCK) promoter (5–7); and expression of the GLUT2 glucose transporter (8), *etc.* A combination of constructs with modified insulin gene and suitable gene delivery vectors has demonstrated partial success in the generation of glucose-modulated insulin secretion. Unfortunately, despite extensive work elaborating on insulin gene therapy for diabetes, limited *in vivo* data on metabolically controlled insulin secretion are currently available.

Insulin is produced in pancreatic islet β-cells from proinsulin by proteolytic cleavage of disulfide bonds of A- and B-chains and the C-peptide (9,10). Conversion of proinsulin to mature insulin requires two prohormone convertases in the pancreatic islets. For non-endocrine cells lacking endoproteases, cleavable constructs containing furin (11), a Golgi-associated propeptide endoprotease, have been employed to facilitate processing of proinsulin to active, mature insulin for gene therapy of diabetes (12–14). Exocrine cells in the gastrointestinal system, such as hepatocytes, have been considered for use as surrogate pancreatic islet cells for insulin gene therapy due to their ability to synthesize several enzymes and transporters involved in glucose catabolism. Studies however have shown that distinct factors are involved

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ABBREVIATIONS: dbcAMP, dibutyryl cyclic adenosine monophosphate; DM, diabetes mellitus; EGFP, enhanced green fluorescence protein; IDDM, insulin-dependent diabetes mellitus; IPGTT, intraperitoneal glucose tolerance test; PEI, polyethylenimine; rAAV, recombinant adeno-associated virus; STZ, streptozotocin.

in regulating transcription of glucokinase, the glucose sensor of the products of glucose catabolism (15), in pancreatic and hepatic tissues (16). Regardless of the differences seen when comparing liver cells and pancreatic islets in terms of glucokinase transcription and signaling, hepatocytes possess several desired properties for being considered as surrogate pancreatic β-cells (17), including the expression of glucose transporter GLUT2 and glucokinase for sensing glucose signaling (18).

Adeno-associated virus (AAV) type 2 is a non-enveloped icosahedral non-pathogenic human parvovirus with a single-stranded DNA genome of approximately 4.7 kb. Recombinant AAV-based (rAAV) vectors are being developed as effective delivery vectors for gene therapy (19). The virus exhibits many interesting features for gene transfer such as diverse organ tropism, the capacity to transduce a variety of cells, site-specific integration, and no known pathogenicity. A recombinant AAV vector, harboring the reporter gene under the control of rat insulin I gene promoter, was constructed previously and used to study glucose responsiveness of transgene expression (20). The 5′-flanking DNA (rInsI promoter) of rat insulin I gene contains multiple *cis*-acting transcriptional elements that direct the transcriptional response of glucose (21), resulting in glucose-modulated expression of reporter genes in murine pancreatic islet cells and human hepatoma cells (6,20,22). Conversion of proinsulin to mature human insulin in the pancreatic islets requires the presence of prohormone convertases (PC3/PC1 and PC2) to excise the C-peptide from the A- and B-chains for generation of mature insulin (23,24). Because this regulatory machinery is absent in the liver, the human insulin gene has been mutated to convert the proteolytic processing site to a furin-recognition site, such that normal processing of insulin in non-islet cells is allowed (25). The present study used a genetically engineered vector for generation of rAAV, harboring a furin-mutated insulin gene under the control of a 410-bp rat insulin I promoter (15) to investigate the feasibility of glucose-modulated insulin gene therapy for diabetes *in vivo*. In view of the large quantities of rAAV needed for *in vivo* transduction, the virus was complexed with polyethylenimine (PEI) prior to administration to the livers of the study animals in order to enhance transduction efficiency. Transduction of cells with the hybrid of non-viral and viral vectors, formed by complexation of rAAV with polycations such as protamine or polyethylenimine (PEI), has been previously shown to result in the enhancement of gene transfer (26,27).

In view of the relatively little data available on metabolically modulated *in vivo* insulin gene therapy, this study attempts to examine the glucose- and metabolically controlled insulin secretion, mediated by tissue-specific rAAV vectors, and the potential amelioration of hyperglycemia in streptozotocin (STZ)-induced diabetic animals.

MATERIALS AND METHODS

Construction of a rAAV Vector Containing Furin-Cleavable Insulin Gene

Construction of the rAAV vector used in this study, as described previously (26), involved modification of the plasmid vector pAAV.Ins.Luc.ΔEGFP (20). This plasmid contains the luciferase gene driven by the rat insulin I promoter (rInsI promoter), and the open reading frame (ORF) of the enhanced green fluorescence protein (EGFP), under the control of the immediate early (IE) gene promoter of human cytomegalovirus (CMV). Briefly, plasmid pAAV.Ins.Luc.ΔEGFP was digested with Hind III, blunt-ended, and digested with XbaI to remove the luciferase gene. The plasmid pBAT16.hInsG1.M2 containing the furin-cleavable human insulin gene (hInsM2), mutated between peptides C and A (25), was kindly provided by M. S. German. The 1.3 kb fragment encoding the mutated human insulin gene was digested with Bgl II and Xba I, gel-purified, and inserted into the 6.5 kb AAV vector under the control of rat insulin I promoter. The expression cassettes were placed in series between the inverted terminal repeats (ITRs) sequences of AAV. A schematic illustration of the construct pAAV.rIns1.InsM2.ΔEGFP is shown in Fig. 1.

Preparation of Recombinant Adeno-Associated Virus

Recombinant AAV was prepared by the non-viral method (28). Briefly, rAAV.rIns1.InsM2.ΔEGFP, was prepared by co-transfection of pAAV.rIns1.InsM2.ΔEGFP, containing the furin-mutated human insulin gene under the control of rat insulin I promoter, and pDG (28), containing all AAV and Ad functions required for amplification and packaging of AAV, in 293T cells by the calcium phosphate method. At 72 h post-transfection, cells were centrifuged and dispersed in TD buffer, containing 140 mM NaCl, 5 mM KCl, 0.7 mM K_2HPO_4 and 25 mM Tris–HCl (pH 7.4), followed by four freeze-and-thaw cycles to release the virus. Recombinant AAV was prepared by double CsCl density-gradient ultracentrifugation. Fractions with a refractive index between 1.369 and 1.373 were collected and dialyzed against 0.9% (*w*/*v*) NaCl.

The genomic titers of the rAAV stocks, expressed as numbers of viral particles (pt) per microliter, were determined by dot-blot hybridization as previously described (6,20,22,27). The random primer labeling system (Life Technologies, Inc., Gaithersburg, MD) was used to prepare [α-^{32}P]-dCTP labeled DNA probes, and signal intensities were compared with that of control plasmids.

Glucose-Modulated Insulin Production *In Vitro*

To examine the glucose-responsive production of human insulin, approximately 1×10^6 Huh7 hepatoma cells were seeded into six-well plates and infected for 4 h with rAAV at an MOI (multiplicity of infection) of 500, and incubated at 37°C. At 48 h post-transduction, cells were washed with phosphate buffered saline (PBS), and maintained in glucose-free DMEM containing 10% dialyzed serum and desired

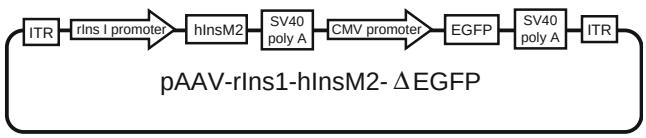

Fig. 1. Schematic illustration of the rAAV construct, pAAV.rIns1.InsM2.ΔEGFP, containing the furin-mutated human insulin gene under the control of rat insulin I promoter.

concentrations of glucose. At 24 h post-treatment, cells were analyzed by flow cytometry for EGFP expression, and the culture medium was collected and analyzed for human insulin content by radioimmunoassay.

The Effect of dbcAMP, Theophylline, and Forskolin on rAAV-Mediated Gene Transfer

To investigate the effect of several insulin secretagogues on insulin production after rAAV transduction, Huh7 hepatoma cells were seeded in 6-well plates and transduced with rAAV at an MOI of 500. At 48 h post-transduction, the culture medium was replaced with glucose-free medium containing various concentrations of the insulin secretagogues, including dibutyryl cyclic adenosine monophosphate (dbcAMP), theophylline, and forskolin. Cells were maintained in glucose-free DMEM, supplemented with 10% dialyzed serum and either 1 or 25 mM glucose. After 24 h incubation, the cell culture media were collected, and levels of human insulin production were analyzed by radioimmunoassay (RIA).

The Effect of Polyethylenimine (PEI) on rAAV-Mediated Gene Transfer

To examine the effect of polyethylenimine (PEI) on rAAV-mediated gene transfer, the rAAV virus was pre-mixed with PEI (2 μg/ml), and 5×10^5 Huh7 cells in the six-well plates were infected with rAAV-PEI complexes for 4 h, washed with PBS, and incubated in complete medium for 48 h. The medium was removed and replaced with glucose-free medium containing either 5 or 25 mM glucose for another 24 h, followed by analysis for the production of human insulin.

Radioimmunoassay (RIA) for Human Insulin

The amount of human insulin in the cell culture medium was quantitatively determined using a human insulin specific RIA kit (Cat No. HI-14K, Linco Research, MO, USA), utilizing ^{125}I-labeled human insulin anti-sera in a competitive binding assay. According to the manufacturer, this assay does not cross-react with human proinsulin (<0.2%). The level of radioactivity was measured in a WIZARD 1470 gamma counter (LKB-Pharmacia, USA.).

Induction of Diabetes in Animals using Streptozotocin (STZ)

Male C57BL/6J mice were provided with standard rodent chow and water ad libitum. At 5–6 weeks of age, diabetes was induced in the animals by two intraperitoneal (i.p.) injections of streptozotocin (5 mg/ml, dissolved in 50 mM citrate buffer, pH 3.5) at 125 mg/kg body weight. The control mice were injected with 0.9% NaCl. On day 7 and day 14 after STZ treatment, tail blood was obtained from the animals after 8 h of fasting, and serum glucose was determined using a One-Touch II portable blood glucose monitor (LifeScan, Milpitas, CA). Animals with blood glucose levels between 250 and 550 mg/dl were considered diabetic and used for the experiments. All experimental procedures on the animals were approved and were in compliance with the guidelines of the institutional review board.

Infection of Animals with rAAV

Mice were anesthetized by i.p. injection of chloral hydrate at 400 mg/kg, followed by abdominal incision. Recombinant AAV, at 3×10^{11} rAAV particles per kg body weight, was complexed with polyethylenimine 25K (PEI-25K) at a final concentration of 2 μg/ml in a volume of 300 μl and injected into the liver by a single administration, using a 1-ml syringe and a 30-gauge needle. The wound was then sutured for recovery. The control group received the same volume of 0.9% NaCl. Animals were routinely monitored for changes in body weight, blood glucose levels, and human insulin secretion thereafter. STZ-induced diabetic mice without rAAV treatment served as the diabetic control group. Blood samples were collected from the tail vein and stored at −20°C before assay. Experiments were carried out using at least three animals per group.

Intraperitoneal Glucose Tolerance Test (IPGTT)

In vivo glucose-modulation of rAAV-PEI mediated insulin gene therapy was examined in the healthy control and STZ-induced diabetic animals. On day 78 and day 104, an intraperitoneal glucose tolerance test (IPGTT) was carried out after 8 h of fasting. Animals were i.p. injected with 1.5 g/kg body weight of glucose, with or without theophylline (50 mg/kg). Tail blood samples were collected every 30 min for up to 150 min after glucose administration, followed by determination of the blood glucose level by a portable One-Touch II blood glucose monitor (LifeScan Inc., Milpitas, CA). Serum separated from the blood samples after centrifugation was frozen at −20°C before assay for the human insulin content.

Immunohistochemical Staining

On day 150 after rAAV treatment, two mice were fasted for 8 h and blood glucose was determined to be 137 and 134 mg/dl, respectively. One mouse with blood glucose of 137 mg/dl (low glucose) was sacrificed and the liver removed, and the other one with blood glucose of 134 mg/dl was i.p. injected with glucose at 1.5 g/kg, and sacrificed 1 h later when the blood glucose reached 342 mg/ml. Both animals were anesthetized by intraperitoneal injection of chloral hydrate (400 mg/kg), and perfused with normal saline containing 4% paraformaldehyde. Liver sections were fixed in 10% (*v*/*v*) neutral formalin for 24 h and embedded in paraffin. The paraffin-embedded tissue sections were deparaffinized and rehydrated in descending concentrations of ethanol. Sections were incubated with mouse anti-human insulin antibody overnight (Sigma Chemicals, MO), washed with PBS, and incubated with biotin-labeled goat anti-mouse Ig and streptavidin-conjugated horseradish peroxidase (HRP; LSAB kit, Dako, Denmark). Section of human pancreas, obtained from National Taiwan University Hospital, was used as the positive control specimen for immunohistochemical staining. HRP activity was developed with a buffered 3-3″-diaminobenzidine tetrahydrochloride (DAB) chromogen solution, followed by examination using a Zeiss Axiophot 2 microscope.

Statistical Analysis

The experimental data were analyzed either by Student's *t* test or one-way ANOVA.

RESULTS

Dose-Dependent Glucose Induction in rAAV-Transduced Huh7 Cells

Huh7 cells were transduced with rAAV (MOI=500), containing the human insulin gene driven by the rat insulin I gene promoter. Transduction of Huh7 human hepatoma cells by rAAV *in vitro* resulted in a glucose-responsive production of immunoresponsive human insulin in the cell culture medium (Fig. 2a). The expression of EGFP in rAAV-transduced Huh7 cells, on the other hand, varied slightly due to better growth conditions at higher glucose concentrations. The maximum increase of geometric mean fluorescence intensity, occurring at 10 mM glucose, was appro ximately 1.4-fold higher than those without glucose treatment (Fig. 2b), compared to the 4.1-fold increase in the production of human insulin at this glucose concentration (Fig. 2a). These data indicated that glucose responsiveness was intrinsically derived from rat insulin I promoter. Maximal production of immunoreactive insulin, obtained at 20 mM glucose, was 148.3±5.5 μU/ml (Fig. 2a), which was approximately 5.6-fold higher than that obtained without glucose treatment. The production of human insulin correlated well with millimolar range of glucose, with the strongest response occurring between 4 and 10 mM glucose.

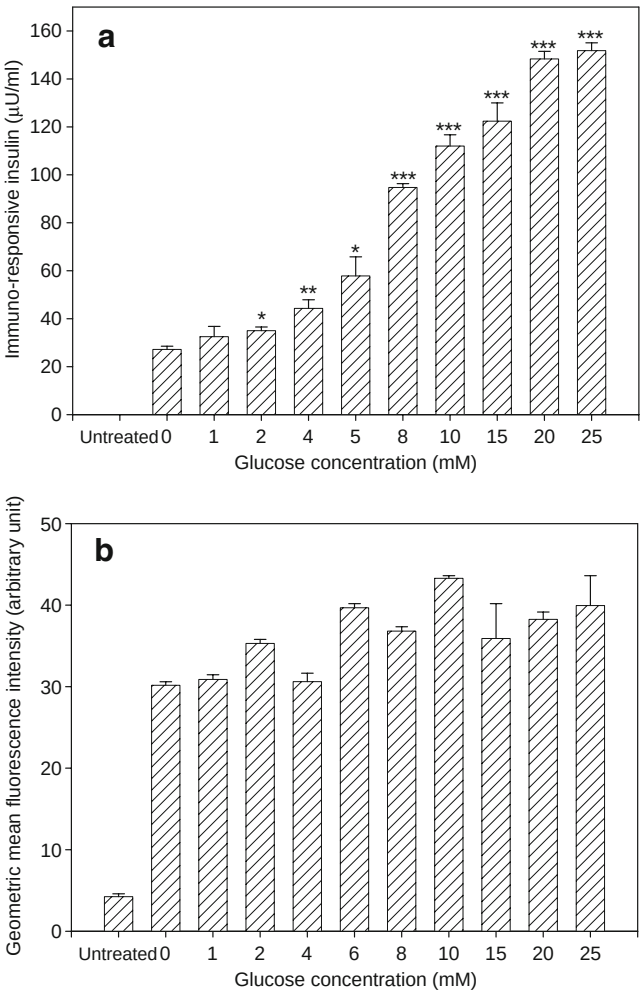

Fig. 2. Effect of glucose concentration on (**a**) the production of human insulin and (**b**) geometric mean fluorescence intensities of EGFP in rAAV-transduced Huh7 cells. Detection of immunoreactive human insulin was performed by radioimmunoassay (*RIA*), and MFI was determined by flow cytometry using a FACSCalibur. *Asterisk* indicates significant difference from the control group without glucose treatment (*$P<0.05$; **$P<0.01$; ***$P<0.001$, by one-way ANOVA).

Effect of dbcAMP, Theophylline and Forskolin on Insulin Production in rAAV-Transduced Huh7 Cells

The stimulating effect of dibutyryl cyclic AMP (dbcAMP), a membrane-permeable cAMP analogue, on the secretion of human insulin in rAAV-transduced cells is illustrated in Fig. 3a. The dependence of insulin secretion on dbcAMP concentration was comparatively higher for cells incubated at 25 mM glucose than those in 1 mM glucose. Secretion of immunoreactive insulin was dramatically elevated between 200 μM and 2 mM dbcAMP at 25 mM glucose. A maximal immunoreactive insulin level, obtained at 2 mM dbcAMP, was approximately 2.9-fold higher than control cells without dbcAMP treatment.

Theophylline inhibits cyclic nucleotide phosphodiesterase enzymes (PDEs), which catalyze the breakdown of cAMP and cGMP. Inhibition of PDEs leads to an accumulation of cAMP and cGMP, thereby increasing signal transduction. The presence of theophylline thus up-regulates the production of insulin. When treated with 8 mM theophylline at 25 mM glucose, insulin production in the rAAV-treated cells was approximately 1.7-fold higher than those without theophylline treatment (Fig. 3b). A maximal secretion level of 217.86 μU/ml mature human insulin was obtained at 8 mM theophylline in the presence of 25 mM glucose.

Treatment of rAAV-transduced Huh7 cells with forskolin (29), an adenylate cyclase activator, also resulted in elevation of insulin production, reaching a maximum at 20 μM forskolin in the presence of 25 mM glucose (Fig. 3c). Although forskolin generated a weaker stimulatory effect at 1 mM glucose, a 2.9-fold increase in human insulin production over the untreated control cells was observed at 25 mM glucose (Fig. 3c).

Variation of insulin levels at 25 mM glucose in the absence of insulin secretagogues was observed, which was attributed to the variability of cell conditions and infectivity of rAAV, resulted from different batches of virus preparations, since genomic titers, rather than infectious titers, were employed in this study for infection. Each individual experiment reported herewith, however, was carried out independently and results were expressed as the means±SEM, based on those obtained from the same batches of cells and viruses. The decline in the secretion of immunoresponsive insulin at higher concentrations of insulin secretagogues, as observed in Figs. 3a–c, was speculated to be caused by the cytotoxicity of dimethylsulfoxide (DMSO), the solvent for dbcAMP and forskolin, and partly by the drugs at these concentrations.

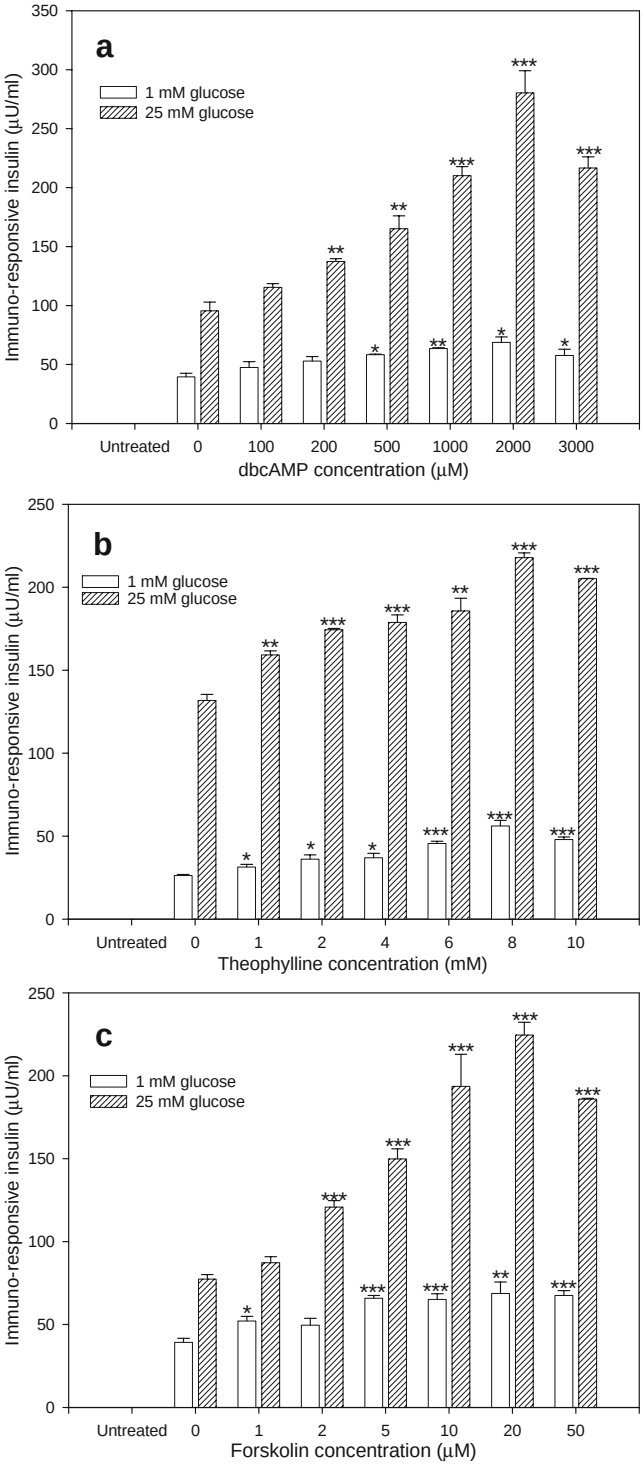

Fig. 3. Effect of dbcAMP, theophylline, and forskolin on rAAV-transduced Huh7 cells in the presence of 1 or 25 mM glucose. Cells were stimulated with varying concentrations of (**a**) dbcAMP, (**b**) theophylline, or (**c**) forskolin for 24 h, and levels of human insulin were determined. Non-transduced cells served as the negative control. Results shown are means±SEM of three separate experiments. *Asterisk* indicates significant difference from the untreated control group (*$P<0.05$; **$P<0.01$; ***$P<0.001$; by one-way ANOVA).

The Reversibility of Glucose-Stimulated Insulin Secretion

To confirm the reversibility of glucose-stimulated insulin secretion, Huh7 cells were infected with rAAV at an MOI of 500. The transfected cells were incubated in glucose-free medium for 4 h before insulin assay experiments. On day 1, the test group and the control cells were treated with 5 mM glucose, and the culture medium was collected 24 h later. Cells were then divided into two groups (A and B). Cells in the group A were treated with 25 mM glucose, while cells in group B were treated with 5 mM glucose. On day 3, the glucose concentration in both groups was alternated with group A receiving 5 mM glucose and group B receiving 25 mM glucose. On day 4, both groups were treated with 5 mM glucose. The culture medium was collected daily and assayed for human insulin content using the RIA kit. Results shown in Fig. 4 demonstrate that rAAV-transfected Huh7 cells secreted basal levels of human insulin of 34.1±2.5 μU/ml at 5 mM glucose. Human insulin levels were elevated significantly up to 87.7±7.1 μU/ml upon stimulation with 25 mM glucose. The production of human insulin was shown to be reversible when the glucose concentrations used to stimulate the cells were reversed. These results demonstrate the effectiveness of the insulin I promoter on glucose regulation in Huh7 hepatoma cells.

The Effect of Polyethylenimine (PEI) on rAAV-Mediated Gene Transfer

Secretion of human insulin in response to glucose concentration was re-examined in Huh7 cells transduced with rAAV-PEI complexes. Results shown in Fig. 5 illustrated that complexation of rAAV with PEI enhanced rAAV-mediated gene transfer *in vitro*, and that production of human insulin was significantly higher in rAAV-transduced cells at 25 mM

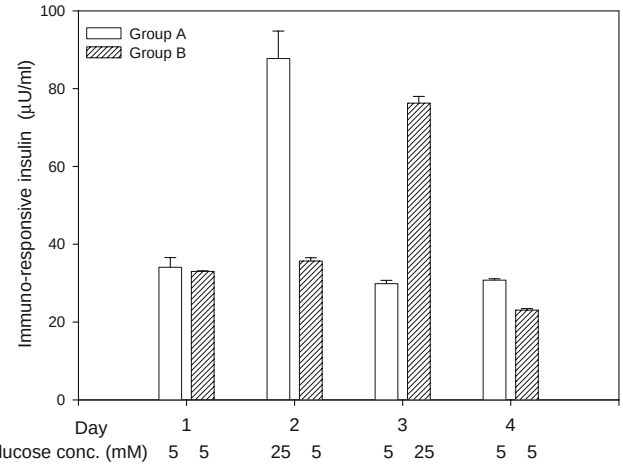

Fig. 4. Reversibility of glucose-stimulation on insulin secretion in rAAV-transduced Huh7 cells. Recombinant AAV-transduced Huh7 cells were divided into two groups (groups *A* and *B*) and treated as follows: Group *A*: 5 mM glucose on day 1, 25 mM glucose on day 2, 5 mM glucose on day 3, and 5 mM glucose on day 4; Group *B*: 5 mM glucose on day 1, 5 mM glucose on day 2, 25 mM glucose on day 3, and 5 mM glucose on day 4. Data of human insulin levels are expressed as means±SEM from triplicate experiments of duplicate wells.

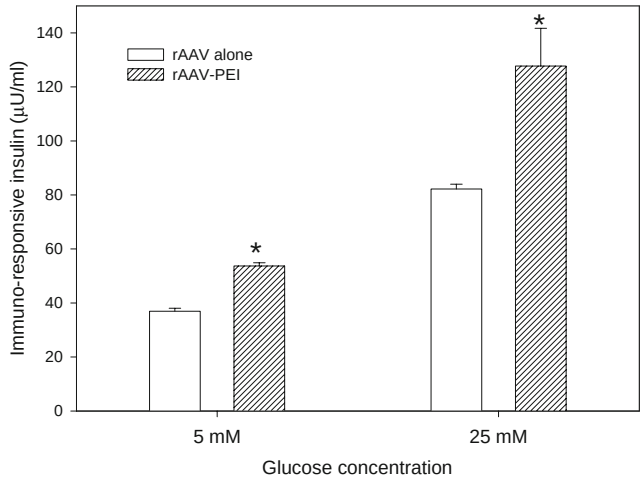

Fig. 5. Effect of PEI on rAAV-mediated gene transfer in Huh7 cells. Huh7 cells infected with rAAV (MOI=500) or rAAV–PEI complexes were given 5 or 25 mM glucose at 48 h post-transduction. The media was assayed 24 h later by RIA for human insulin. Results are given as means±SEM from triplicate experiments. *Asterisk* indicates significant difference from the control cells treated with rAAV alone at the same glucose concentrations ($P<0.05$).

glucose than those at 5 mM glucose, a normal human fasting blood glucose level.

Intraperitoneal Glucose Tolerance Test (IPGTT) and the Effect of Theophylline *In Vivo*

The intraperitoneal glucose tolerance test (IPGTT) was performed on day 78 after rAAV-PEI treatment by fasting the animals for 8 h, followed by intraperitoneal injection of glucose at 1.5 g/kg, and blood samples were taken every 30 min for up to 150 min to determine the glucose levels and secretion of human insulin. Before injection blood glucose levels in rAAV-PEI treated STZ-diabetic mice ranged from 105–170 mg/dl, which rose significantly 30 min after glucose challenge, peaking with the maximum value of 351±49 mg/dl at 60 min, and returned to baseline (158±7 mg/dl) by 150 min after glucose injection (Fig. 6a). On day 104 after rAAV-PEI treatment, the mice were subjected to another IPGTT by co-administration of theophylline, an insulin secretagogue, during glucose challenge. Animals were fasted for 8 h and injected (i.p.) with glucose (1.5 g/kg) and theophylline (50 mg/kg), followed by collection of tail blood samples at multiple time points. Co-administration of glucose and theophylline in rAAV-PEI treated animals raised the blood glucose to 310±22 mg/dl at 30 min, and returned to a lower blood glucose level (178±7 mg/dl) at 60 min following injection. The STZ control group, on the other hand, remained hyperglycemic (404 to 600 mg/dl) throughout the experimental period. Despite reductions in blood glucose, compared to the STZ-control group, no animal treated with rAAV–PEI complexes succumbed to hypoglycemia during the course of this study.

Comparison of the blood insulin levels in the groups treated with glucose–theophylline and those with glucose alone illustrated some interesting features. No human insulin was detected in the healthy or STZ-induced diabetic control groups; whereas production of human insulin was significantly increased in the rAAV-PEI treated animals 30 min after injection, peaking at an insulin level of 14.4±0.5 μU/ml 1 h after glucose injection, and decreased thereafter (Fig. 6b). In contrast, co-administration of glucose and theophylline in rAAV-PEI treated mice dramatically enhanced the secretion of human insulin. The blood insulin value at 30-min post-injection was 24.2±1.3 μU/ml, which rose slightly to a maximum of 25.2±0.8 μU/ml at 60 min after injection (Fig. 6b), approximately 1.75-fold higher than that of the group treated with glucose alone. These results demonstrated that co-administration with glucose and theophylline enhanced the secretion of human insulin, resulting in a faster decrease in blood glucose levels, compared to those animals exposed to glucose alone.

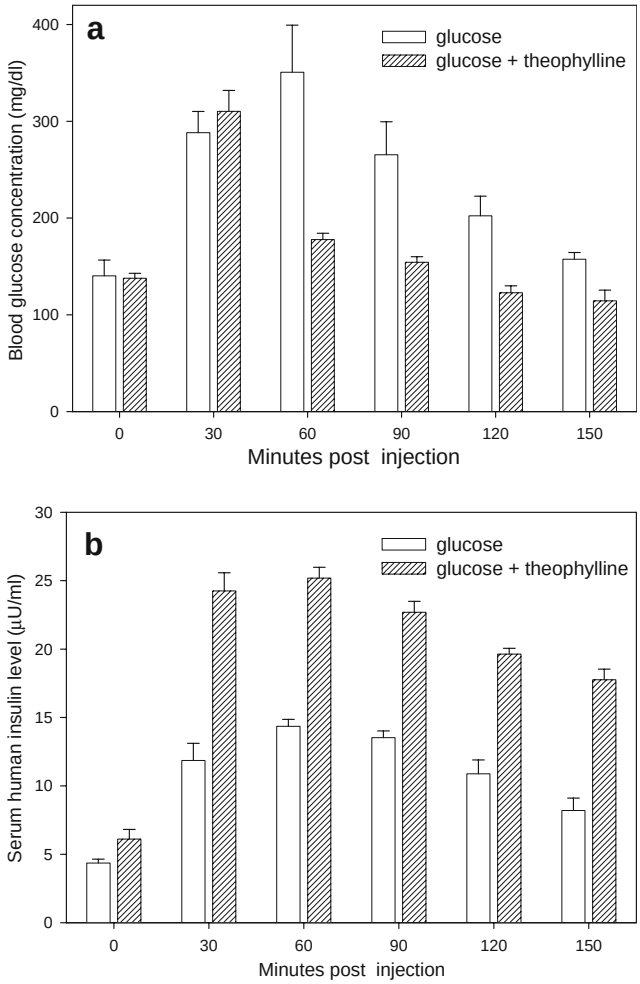

Fig. 6. Effect of glucose and theophylline on human insulin secretion and blood glucose levels in the animals. Intraperitoneal glucose tolerance test (IPGTT) was carried out on day 78 after rAAV–PEI treatment, and the time-course changes in blood glucose and serum immunoreactive human insulin levels in the animals were followed. On day 104 after rAAV–PEI treatment, the animals were subjected to another glucose tolerance test by intraperitoneal co-administration of glucose and theophylline. Data presented are time-course changes of (**a**) blood glucose levels, and (**b**) serum immuno-reactive human insulin levels, after treatment with glucose alone (open columns) or co-administration of glucose and theophylline (*hatched columns*). Results shown are the means±SEM for each group ($n\geq3$).

Immunohistochemical Detection of Human Insulin Production in the Liver of rAAV-Transduced Animals

Immunohistochemical staining of liver sections shown in Fig. 7 demonstrate that incorporation of PEI enhanced transgene expression and that animals treated with rAAV–PEI complexes secreted higher levels of human insulin. However, there was no significant difference in the levels of insulin production between the animals exhibiting high (342 mg/dl) and low (137 mg/dl) blood glucose levels. The failure to detect a significant difference in insulin expression in the liver between animals with high and low blood glucose could likely be explained by the secretion of mature insulin into the blood circulation from rAAV-transduced hepatocytes.

DISCUSSION

Glucose-modulated insulin gene therapy aims to regulate production of biologically active insulin with blood glucose level in order to reduce the frequency of insulin administration. One of the major challenges in insulin gene therapy for type I diabetes mellitus was to develop effective insulin secretion in response to physiological levels of glucose metabolites. Although considerable efforts have been made towards glucose-modulated insulin therapy, aiming at production of insulin in the non-beta cells, limited *in vivo* experimental data showing glucose-regulated insulin production were available to date. Continuing previous studies on glucose-modulated rAAV-mediated gene transfer (20,22,27), rAAV encoding furin-mutated human insulin, under the control of the rat insulin I gene promoter, was constructed. The construct was used in the present study to examine glucose- and metabolically modulation of insulin gene therapy for insulin-dependent diabetes.

In vitro experiments on glucose responsiveness performed here demonstrate that rAAV-transduction of Huh7 cells, a human hepatoma cell line previously shown to possess several endocrine pancreatic features (30), resulted in secretion of immunoresponsive insulin in response to glucose up to 20 mM (Fig. 2). The level of secretion falls within a comparable range reported by others for rat hepatocytes and pancreatic islet cells (15,31,32).

Insulin plays a key role in glucose homeostasis. Secretion of insulin in the pancreatic islet cells is not only controlled by glucose but by other signals, such as cAMP-associated factors (33). Glucose responsiveness regulated through transcriptional control by the rat insulin I gene promoter was examined in this study by stimulating hepatocytes with several insulin secretagogues, including dibutyryl cyclic adenosine monophosphate (dbcAMP), a cell-membrane permeating cAMP, theophylline, a phosphodiesterase inhibitor, and forskolin, an adenylate cyclase activator. Results in Fig. 3, showing the potentiation of insulin secretagogues on insulin production in rAAV-transduced Huh7 hepatocytes, illustrate a synergistic effect of metabolic control of insulin gene transcription in addition to glucose modulation. Glucose responsiveness

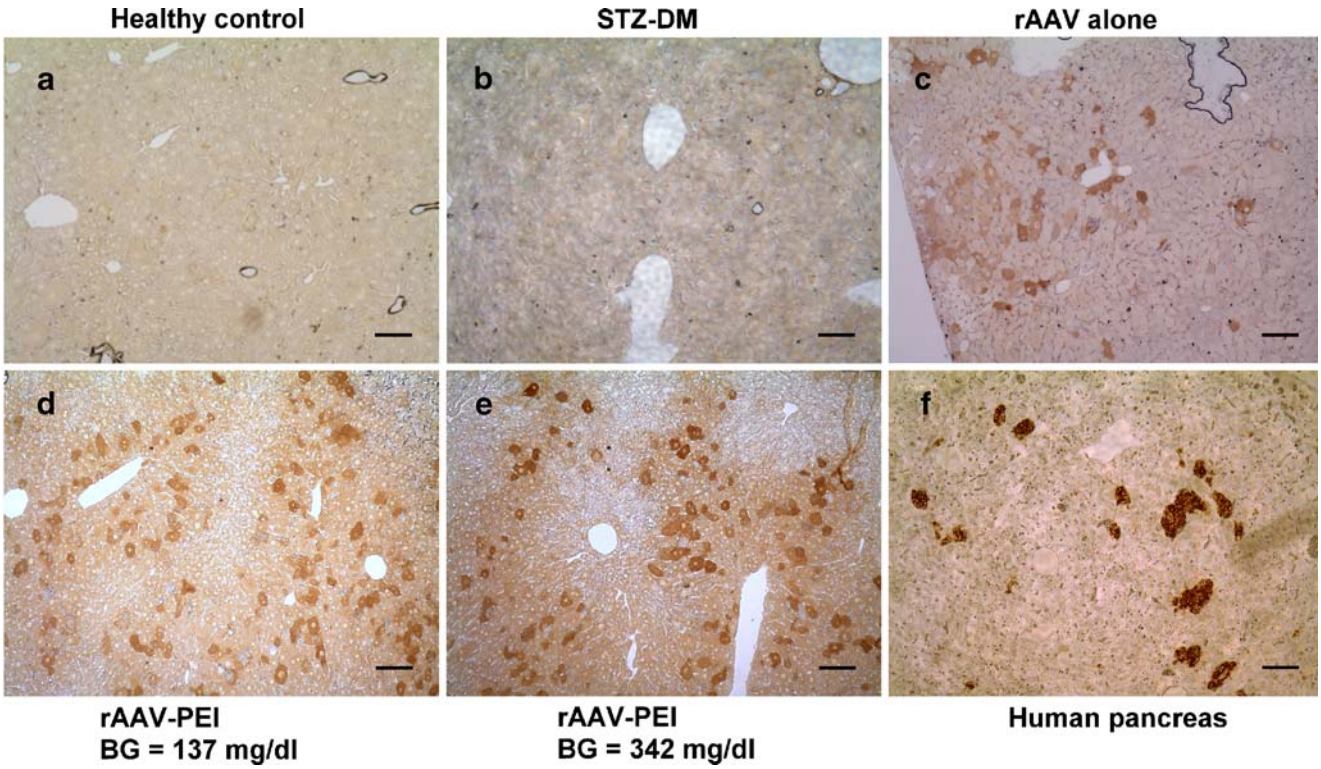

Fig. 7. Immunohistochemical detection of human insulin in mouse livers following rAAV-mediated gene transfer. On day 150 after rAAV–PEI treatment, animals were sacrificed either with or without glucose challenge, and the dissected livers were examined by immunohistochemical detection of human insulin. Results presented are (**a**) healthy control animals, (**b**) STZ-induced diabetic mice without rAAV treatment (negative control), (**c**) STZ-induced diabetic mice treated with rAAV alone, (**d**) rAAV-PEI treated diabetic mice with blood glucose of 137 mg/dl right before sacrifice, (**e**) rAAV-PEI treated diabetic mice with blood glucose of 342 mg/dl 1 h after intraperitoneal injection of 1.5 g/kg glucose, and (**f**) human pancreas, serving as the positive control. Scale bar=50 μm.

regulated by the insulin promoter was further confirmed by the reversibility of glucose-stimulated insulin secretion in rAAV–PEI-treated Huh7 cells (Fig. 4). These results demonstrate the synergistic interaction between hormonally regulated cAMP-dependent signaling and glucose-regulated signaling in the rAAV-transduced human hepatoma cells. Results presented thus far indicate the feasibility of using hepatocytes as surrogate islet cells for insulin gene therapy.

In pursuit of glucose-responsive insulin replacement therapy in ectopic cells, various attempts have been made to couple circulating insulin levels to serum glucose concentrations. However, most studies to date have focused on the "on-" and "off-" *in vitro* regulation of insulin production, and *in vivo* studies mostly emphasized the feasibility of controlling hyperglycemia by transcriptional control of insulin secretion. Limited success has been obtained so far in regulating the *in vivo* secretion of insulin in response to glucose and metabolic signals in ectopic cells. The results presented here on the intraperitoneal glucose tolerance test (IPGTT) in rAAV–PEI-treated animals show that glucose challenge stimulates secretion of human insulin in the PEI–rAAV-treated animals and significantly ameliorates hyperglycemia in the STZ-induced diabetic mice. In contrast, the untreated STZ-diabetic control group remained hyperglycemic (data not shown). A lag time of approximately 30 min for resuming normoglycemia was usually observed in the rAAV–PEI-treated group after glucose challenge, as compared to the healthy control mice, suggesting that transcriptional regulation in the rAAV–PEI transduced animals is relatively slow in stimulating insulin production. Despite reductions in blood glucose compared to the untreated STZ-control group, no animal treated with rAAV–PEI succumbed to hypoglycemia.

Co-administration of glucose and theophylline stimulated even greater secretion of human insulin compared to animals challenged with glucose alone, illustrating a synergistic effect of co-treatment with glucose and theophylline on insulin secretion. Two hours after co-challenges with glucose and theophylline, blood glucose levels resumed to 123±7 mg/dl, close to the normal physiological concentration of blood glucose (Fig. 6a), and the serum human insulin level was approximately 4.4-fold higher than the basal level (Fig. 6b). This shows the reversal of hyperglycemia and ectopic secretion of biologically active insulin in the rAAV–PEI-treated STZ-diabetic animals. Immunohistochemical detection in liver sections further confirms the ectopic expression of human insulin after treatment with rAAV–PEI complexes and the enhancing effect of PEI complexation on transgene expression (Fig. 7). These experimental results strongly demonstrate the effect of the *in vivo* control of rat insulin I gene promoter on glucose responsiveness mediated by rAAV–PEI in STZ-induced diabetic animals.

Although rAAV vectors appear to be less likely to generate host immune responses, precautions must be taken when using the hybrid of non-viral and viral vectors for gene delivery, as we cannot rule out their potential immunogenicity and toxicities. Studies of the PEI-associated cytotoxicity showed that branched and linear PEI can both induce membrane damage and initiation of apoptosis (34). Recombinant AAV-encoded transgene product, on the other hand, has been shown to be immunogenic, depending on the route of administration (35), and immunosuppression might be employed to circumvent the development of anti-AAV immunity (36).

Taken together, this study has demonstrated the feasibility of controlling blood glucose in STZ-induced diabetic mice by rAAV-mediated hepatic insulin gene delivery. Recombinant AAV-PEI mediated hepatic insulin gene therapy, under the control of a glucose-responsive element, was shown to induce both glucose- and metabolically regulated secretion of human insulin and amelioration of hyperglycemia *in vivo.*

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