Insulin expression in livers of diabetic mice mediated by hydrodynamics-based administration

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INTRODUCTION
Type 1 or insulin-dependent diabetes mellitus is resulted from autoimmune destruction of the pancreatic β-cells[1]. Due to severe deficiency of insulin, the key pancreatic hormone necessary for glucose homeostasis, patients with type 1 diabetes suffer from elevated blood glucose levels manifested as thirst, diuresis, overeating, ketoacidosis as well as weight loss. Development of long-term diabetic complications, such as nephropathy, retinopathy, neuropathy and macrovascular disease is also very dangerous and lethal. Currently, type 1 diabetic patients are treated with twice-daily insulin injection, which neither provides adequate glycemic control nor prevents the development of diabetic complications. Although oral and inhaled forms of insulin or insulin pump are used to achieve better glycemic control, these strategies need to be further improved[2].

Insulin gene therapy, an alternative strategy to improve glycemic control in type 1 diabetes, can restore endogenous insulin production by insulin gene delivery. Although regulated insulin expression has been developed recently, the slow kinetics of insulin secretion limits its application in clinical research. On the other hand, basal insulin production has been proved to be an effective and adjuvant strategy in insulin therapy[3]. The most intensely studied method for gene therapy utilizes viruses as a carrier to mediate long-term transgene expression, which is necessary for the chronic nature of diabetes, with improved safety profiles. Of the currently available gene delivery vehicles, only a few viral vectors, including lentiviral and adeno-associated viral (AAV) vectors meet these requirements. Except limited packaging size and the possibility of insertional mutagenesis following delivery of recombinant vectors, viral carriers usually require laborious procedures for preparation and purification[4-5]. All these shortcomings make the viral carriers inconvenient in studying the function of gene products or the practicability of gene expression cassettes.

One of the alternatives currently under development is the direct use of naked DNA that is routine in preparation and safe after administration. But naked plasmid has obstacles in diabetic gene therapy because of its less efficiency to transfect animal cells. Low level expression of insulin fails to reduce blood glucose levels significantly[6-7]. Liu and Zhang have developed a hydrodynamics-based procedure to mediate efficient expression of transgenes in the liver of mice by systemic administration of naked DNA[8-9]. The liver, a surrogate organ for insulin gene transfer in most preclinical tests in vivo, showed the highest level of gene expression among the organs expressing transgenes. This strategy has been proved to be a convenient way in studying gene function and gene therapy. We used the hydrodynamics-based procedure, a convenient and safe strategy for naked DNA transfer, in the study of diabetes gene therapy.

In this report we injected naked plasmid with insulin precursor cDNA into diabetic mice via hydrodynamics-based procedure. The basal insulin production and its effect on the reversal of diabetic manifestations were evaluated. Sleeping...
**MATERIALS AND METHODS**

**Construction of plasmid vector**

Engineered human proinsulin cDNA (mHINS) contained furin recognition sequences (Arg-X-Arg-Arg) at B/C and C/A junction regions. Such a genetic modification allowed proinsulin to be processed maturely by the ubiquitous protease furin in non-endocrine cells. An expression cassette containing mHINS cDNA driven by a CMV promoter in plasmid pcDNA3-mHINS (provided by Dr. Shen KT in Zhongshan Hospital) was recloned between two inverted reverse sequences in plasmid pT, a Sleeping Beauty transposon vector (provided by Dr. Zoltan Ivics). Plasmid used for transfection was purified with a purification kit (Qiagen) according to the instructions.

**Animal studies**

Male ICR mice (from BK Company, weighing 25-30 g) were used in this study. All the mice were fed at room temperature with sufficient water and food, and monitored for body weight every 3 days. To induce diabetes, the animals received intraperitoneal injection of streptozotocin (STZ dissolved in a 100 mMol/l sodium citrate solution pH 4.5, 150 mM NaCl, immediately before administration) at the dose of 150 mg/kg body weight. The mice with blood glucose levels in the range of 20-30 mMol/l were selected and used 7 days after STZ treatment. Blood glucose levels were determined with Precision Plus Electrodes (Medisense, MA). Plasma insulin levels were measured by human insulin radioimmunoassay (RIA, Linco Research, St. Louis, MO). Blood samples were collected by a retro-orbital technique into Eppendorf tubes. Subsequently, separated plasma from each of two to three mice in each group was mixed to obtain enough volume of samples for insulin evaluation assays.

**Plasmid administration**

Administration of mHINS vectors into STZ-induced diabetic mice was carried out by intravenous coinjection of 80 µg of pT-mHINS and 8 µg of pCMV-SB (plasmid expressing transposase), or intravenous injection of 80 µg of pcDNA3-mHINS in 2-3 ml (one tenth of the body weight in grams) of Ringer’s solution (147 mM NaCl, 4 mM KCl, 1.13 mM CaCl2) in 7 seconds.

**Glucose tolerance test**

The mice were subjected to three hour fasting and injected intraperitoneally with 20% glucose at the dose of 2 g/kg body weight. Blood glucose was measured at 30-minute intervals before and after glucose infusion.

**Identification of mHINS DNA and mRNA in livers**

Genomic DNA prepared from livers of mice was used for PCR detection with primers specific for mHINS cDNA (forward, 5’-CGACGCTTTGTGGAACCA-3’; and backward, 5’-TCCACAAATGCCACCGCTT-3’). Total RNA from livers was reversely transcribed and the resulting pool of cDNA was subjected to PCR detection with the same primers above. The reaction mixture for PCR amplification was subjected to 30 cycles of denaturation (95°C, 30 seconds), annealing (63°C, 30 seconds), and extension (72°C, 30 seconds). The amplified products were 204 bp in length and identified by agarose-gel electrophoresis. For β-actin RT-PCR, the sequences of primers were 5’-CTTCCTCTGGATCGATGCTCT-3’ and 5’-GGAGCAATGTCTGTGACCT-3’. The PCR conditions involved denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s for 30 cycles, and the PCR products were 202 bp in length. Here, β-actin RT-PCR was used as an internal control.

**Quantitative RT-PCR analysis**

On days 1, 7 and 14 after plasmid injection, we sacrificed the mice that had received pCMV-mHINS and pT-mHINS, and harvested the livers. The total RNA of the liver samples was isolated and the contaminated DNA was excluded with DNase I. Two micrograms of quantified RNA were first subjected to reverse transcription using an oligo(dT) primer and reverse transcriptase (RT, Gibco BRL, Rockville, MD, USA) to generate first-strand cDNA. To determine the levels of transgene-derived insulin mRNA in the liver, we performed quantitative real-time PCR analysis using an ABI PRISM 7000 system. Taqman Universal PCR MasterMix was used to provide critical components for PCR mixture. The primers and probe were as follows: forward primer, 5’-AACACCTTGGGCTCAAGA-3’; and backward, 5’-CGTCCCCGCAACTAGTA-3’. FAM labeled MGB probe, FAM-5’-GTGGTGGAAGCTCT-3’-MGB. The PCR process was at 50°C for 2 min, at 95°C for 10 min, then at 95°C for 15 s and at 60°C for 10 min for a total of 40 cycles. The length of expected products was 54 bp. The standard curve for mHINS plasmid was created. The quantity of insulin mRNA was expressed as pg/cDNA from 100 ng of total RNA.

**Immunohistochemical analysis**

For immunohistochemical (IHC) procedures, the tissues were fixed in 4% buffered paraformaldehyde. An antibody to human insulin (Linco Research) was used for immunostaining. The number of insulin-positive hepatocytes was counted on IHC sections from 3-4 animals.

**RESULTS**

**Reduction of blood glucose in the diabetic mice treated with insulin precursor DNA**

The expression constructs of human insulin precursor driven by CMV promoter are shown in Figure 1. Insulin precursor vectors were injected rapidly via tail vein into diabetic mice, and blood glucose of the mice was measured to determine the effect of insulin production after the naked vector delivery. As shown in Figure 2, the administration of insulin vectors resulted in a significant reduction in glucose concentration. In the eight animals co-transferred with pT-mHINS and pCMV-SB, their blood glucose levels were reduced to normal one day after plasmid injection, and three of them maintained the normal levels for two weeks. Glucose concentration was also reduced significantly one day after administration of the non-transposon vector pcDNA3-mHINS, but increased rapidly and reached pretreatment levels one week later. In contrast, Ringer’s solution-treated animals remained the high glucose levels unchanged. The severe loss of body weight of the diabetic mice was ameliorated after delivery of pcDNA3-mHINS and pT-mHINS (Figure 3). Also, urination of the treated diabetic mice lessened dramatically. These results indicated that symptoms of diabetes were improved after hydrodynamics mediated insulin gene delivery. No diabetic mouse died from the rapid tail vein injection, which showed the safety of hydrodynamics-based DNA administration in the study of gene therapy for diabetes.

**Insulin production in STZ-induced diabetic mice after insulin vector treatment**

Insulin protein in mouse serum was measured by radioimmunoassay with human insulin polyclonal antibodies. Normal mice and STZ-induced diabetic mice treated with
Ringer’s solution were used as controls. Insulin protein in pT-mhINS and pCMV-SB co-treated STZ mice was 97.2 µU/ml, 33.3 µU/ml, and 12.7 µU/ml one day, seven days and fourteen days after treatment, respectively. In pcDNA3-mhINS treated mice it was 113 µU/ml, 4.8 µU/ml, and 3.4 µU/ml one day, seven days and fourteen days after treatment, respectively (Table 1). Seven days after plasmid administration, insulin production was seven fold higher in serum of diabetic mice treated with transposon vector than that in serum of those treated with non-transposon vector, indicating transposon vector was more effective to mediate sustained basal insulin production.

Table 1 Insulin production in STZ-induced diabetic mice after insulin vector treatment (mean±SD)

| Serum insulin Level(µU/ml) | pCMV-mhINS+ | pCMV-SB | Diabetic control | Normal control |
|---------------------------|-------------|---------|------------------|---------------|
| 1 day after treatment     | 113.1±20.6  | 97.2±14.4 | 5.2±0.3          | 10-50         |
| 7 days after treatment    | 4.8±1.3     | 33.3±0.2 | -                | -             |
| 14 days after treatment   | 3.4±0.2     | 12.7±6.6 | -                | -             |

Figure 1 Sucture of pcDNA3-mhINS, pT-mhINS, and pCMV-SB. CMV, CMV promoter; mhINS, engineered human insulin cDNA; IR, inverted repeat sequences of Sleeping Beauty.

Figure 2 Reduction of blood glucose in diabetic mice treated with insulin precursor DNA. Data were plotted as the mean ±SEM. P <0.01 (1d). Vector administration was at day 0.

Figure 3 Weight increment of the mice after the mhINS vector treatment. Vectors were administrated at day 0.

PCR and RT-PCR detection for mhINS cDNA and mRNA in livers
Total DNA and RNA were extracted for PCR and RT-PCR to detect if mhINS vectors were transfected into the mice livers and transcribed into mRNA. The PCR and RT-PCR products of 204 bp fragments were amplified from all livers of mice administrated pcDNA3-mhINS and pT-mhINS, but not from controls. In RT-PCR detection with β-actin primers, 202 bp fragments were found in all hepatic RNAs (Figure 4).

Figure 4 PCR and RT-PCR detection for mhINS cDNA and mRNA in livers. A, mhINS cDNA PCR; B, mhINS mRNA RT-PCR; C, β-actin RT-PCR. The number above the photo shows DNA marker (1), positive control (2), negative control (3), livers of pT-mhINS treated mice 1 day (4) and 7 days (5) after administration, livers of pcDNA3-mhINS treated mice 1 day (6) and 7 days (7) after administration.

Quantitative real-time RT-PCR analysis for insulin mRNA in livers
To identify the difference of transcript levels of mhINS in the livers of diabetic mice treated with transposon and non-transposon vectors seven and fourteen days after injection, real-time RT-PCR was performed. The transcripts of mhINS in the livers of mice treated with transposon vector were more than those in the livers of mice treated with non-transposon vector seven days or fourteen days after delivery (Table 2). No mhINS transcripts were detected in the livers of negative control mice. To identify if the isolated RNA was contaminated by mhINS DNA, RNA isolated from the livers of plasmid-treated mice but not reversely transcribed was subjected to real-time PCR. Negative results suggested that the isolated RNA was not contaminated by mhINS DNA.

Table 2 Quantity of mhINS mRNA in mouse liver after plasmid infusion (mean±SD)

| Quantity of mRNA(pg) | pCMV-mhINS | pT-mhINS+ | pCMV-SB | Diabetic control |
|----------------------|------------|-----------|---------|------------------|
| 7 days after         | 0.21±0.14  | 3.41±1.48 | 0       | 0                |
| treatment            |            |           |         |                  |
| 14 days after        | 0.13±0.04  | 1.24±0.06 | 0       | 0                |
| treatment            |            |           |         |                  |

Expression of insulin protein in livers of treated diabetic mice
To confirm that treatment of the diabetic mice with mhINS vectors resulted in insulin expression in the liver, immunohistological staining of liver sections with antibodies against insulin was performed. As shown in Figure 5, insulin-positive cells were observed in all livers of mhINS vectortreated mice. In contrast, it was negative in the livers of Ringer’s solution-treated mice. To provide more quantitative information, liver sections from three cases in each group were randomly observed 7 days and 14 days after plasmid administration and the number of insulin-positive hepatocytes were counted. There were more positive cells in the livers of mice treated with transposon vector as compared with those treated with non-transposon vector (Table 3).
Effects of plasma insulin on glucose tolerance in diabetic mice treated with insulin vectors

To assess whether or not hepatic products of insulin at basal levels had any therapeutic effect on blood glucose disposal, glucose tolerance experiments were performed in the treated diabetic animals. As shown in Figure 6, blood glucose levels in normal control mice moderately rose to about 12 mM after glucose infusion, and rapidly restored to normal within 1.5 h. In contrast, glucose infusion further exacerbated the severity of hyperglycemia in diabetic animals, resulting in higher glucose concentration (over 22 mM), which persisted for more than 2 hours before slow decline. Blood glucose levels of the diabetic mice treated with insulin vectors were moderately elevated to the same degree as that of the normal mice after glucose challenge and returned to pre-challenge levels within 2 hours. These results suggested that sustained expression of hepatic insulin at basal levels could significantly enhance glucose tolerance in type 1 diabetic animals.

**DISCUSSION**

One of the difficulties in gene therapy for diabetes is the requirement of high production of insulin to reduce blood glucose levels. Viral vectors, such as adenovirus[13-15], AAV[16-18], and retrovirus[19] have been used. Productive transduction by retroviruses depends on cell division, which is a limiting factor for transducing postmitotic cells. In contrast, adenoviruses are effective for transducing non-dividing cells, but leaky expression of adenoviral proteins is immunogenic, consequently resulting in transient transgene expression. Recombinant AAV vectors have been widely used for gene transfer because of their inherently non-immunogenic nature and ability to stably transduce both dividing and non-dividing cells. Although rAAV is able to mediate stable transgene expression, efficient transduction of the liver by rAAV vector requires intraportal vein injection instead of peripheral intravenous infusion, and stable transgene expression is detected only in approximately 5% of liver cells. Naked plasmid vector, simpler, cheaper and more routine in preparation than viral vectors, is convenient in studying gene function and the practicability of gene expressing vectors. Hydrodynamics-based procedure can mediate naked DNA transfer to the liver most efficiently. It has been used in the study of transfer and expression of various genes, such as FIX[20], IL-10[21], LDL[22], HDV[23], hepatocyte growth factor[24], etc. The method was proved to be very efficient for delivery of these genes into the mouse liver. The peak level of gene expression could be gained 24 hours after injection[8,9]. Transaminase levels and liver histological results showed that rapid intravenous plasmid injection into mice could induce transient focal acute liver damage, which was rapidly repaired within 3 to 10 days. In the present study, we used hydrodynamics-based procedure to deliver insulin gene into STZ-induced type 1 diabetic mice. Insulin production was greatly improved after plasmid injection, and hyperglycemia was recovered to normal levels at 30 min intervals prior to and after glucose challenge were determined and plotted as a function of time. Data were obtained 2 days after insulin vector treatment. Data are the mean±SEM, n=2 or 3.

Table 3 Insulin-positive cells in mouse liver after plasmid infusion (mean±SD)

| Positive cells | pCMV-mhINS | pT-mhINS | Diabetic control |
|---------------|------------|----------|-----------------|
| 7 days after treatment | 6.3±2.3 | 19.0±5.7 | 0 |
| 14 days after treatment | 2.3±0.9 | 9.3±2.4 | 0 |

**Figure 6** Effects of plasma insulin on glucose tolerance in the diabetic mice treated with the insulin vectors. Blood glucose levels at 30 min intervals prior to and after glucose challenge were determined and plotted as a function of time. Data were obtained 2 days after insulin vector treatment. Data are the mean±SEM, n=2 or 3.
changes in blood glucose concentrations. In addition, the liver is a major insulin target organ for glucose homeostasis and helps to maintain blood glucose concentrations in a narrow physiological range. Another advantageous feature of hepatic insulin production is related to the observation that portal insulin levels are relatively higher than peripheral insulin concentrations, as pancreatic insulin is released directly to portal circulation. Insulin gene expression in the liver is likely to restore to some extent portal/peripheral insulin concentration gradient in type 1 diabetes. Finally, the liver is a relatively large organ with a robust capacity for protein synthesis, and even a small fraction of hepatocytes transduced will be able to secrete sufficient amount of insulin for improving glycemic control in type 1 diabetes. Taken together, the liver is an excellent target site for insulin gene delivery. The limitation that the liver lacks pancreas-specific prohormone convertases PC2 and PC3 for insulin processing has largely been overcome by converting amino acid residues at B/C and C/A junction regions of the proinsulin polypeptide chain to a consensus furin recognition site.[23,24]. So proinsulin synthesized in hepatocytes will be processed by ubiquitously expressed enzyme furin. Hydrodynamics-based administration can efficiently deliver foreign gene into the liver. In this study, furin-cleaved proinsulin gene was injected into diabetic mice via rapid tail vein. There was a high expression of insulin mRNA and protein in the liver of mice treated with mhINS plasmid vectors. The glucose disposal of diabetic mice was also improved. These results indicated that hydrodynamics-mediated insulin expression in the liver was effective to improve the glucose control in type 1 diabetic mice.

One limitation of non-β cell expression of insulin gene is the lack of regulated protein secretory pathway. To overcome this limitation, different approaches have been taken to control hepatic insulin gene expression in the liver. While considerable process has been made to achieve regulated insulin gene expression[25-27], there are several issues concerning the safety and potential toxicity of such systems. A slow kinetics in insulin production and secretion from the liver in these reports was life-threatening.[28]. Sustained basal hepatic insulin production, another effective strategy in therapy of diabetes however, could be used as an adjuvant treatment to insulin therapy, and could confer profound therapeutic benefits to type 1 diabetes mellitus.[29]. Basal insulin represents an important physiological phase of insulin secretion in the post-absorption phase. In addition to providing the basal requirement for hormones in glucose metabolism, basal insulin played a key role in preventing the development of ketoacidosis[30]. In the present study we transferred insulin expression plasmid controlled by a constitutive CMV promoter into STZ-induced diabetic mice. The results showed that non-regulated basal production of plasma insulin was significantly increased after plasmid administration. Blood glucose levels were reduced to normal and glucose tolerance was improved also. Weight loss was also ameliorated. Although the treated mice occurred hypoglycemia during fasting, controlling the dose of DNA injection could overcome it. Basal insulin production, when combined with twice-daily insulin injection, could achieve better glycemic control without the need of multiple daily insulin injections and excessive body weight gain, an inevitable consequence associated with intensive insulin therapy.[30]

Unlike mammalian promoter, CMV promoter delivered in vivo is known to induce cytokine production and cytokine-mediated effects could subsequently attenuate the promoter activity and limit transgene expression.[31,32]. We delivered mhINS under the promoter of rat liver pyruvate kinase gene (LPK) that has glucose response element. However, there was no difference in ameliorating hyperglycemia between two promoter-driven plasmids (data not shown). Elongation factor promoter (EF) was also used to drive insulin expression. Though less DNA was required to normalize glucose concentration, there was no significant difference in prolonging the normalized state of glucose concentration between CMV and EF promoters (reported in another article). Some points would account for these results. A majority of DNA began to lose one day after injection and insulin expression declined below certain levels that can not significantly reduce blood glucose levels. So there was no significant difference in reduction of glucose level among CMV, EF and LPK promoters when the peak level expression of insulin was over. So how to stabilize DNA infused into hepatic cells and how to prevent gene silencing would be the most critical steps before hydrodynamics-based procedure can be used in gene therapy of diseases that need high levels of gene products.

Sleeping Beauty vector, one of mammalian transposon systems, was used in this study to prolong the high levels of insulin expression. Sleeping Beauty transposon could be efficiently inserted into mammalian chromosomes in vivo and might permit longer-term foreign gene expression with a single administration.[33]. The results indicated that the effect of transposon vector on hyperglycemia excelled that of non-transposon vector. Although the feature of unspecific insertion made transposon system unsafe in the practicality of gene therapy, it was still valuable in the study of gene function for diabetes therapy. Other safer systems to prolong naked DNA expression have been developed recently, such as phase θ31 integration system[34], combination of hepatic control region and hepatic promoter, etc.[20,35-37]. These strategies, combined with hydrodynamics procedure, would be very valuable in the study of gene therapy for diabetes.

Although hydrodynamics-based procedure is unsuitable for clinical use, local gene transfer with such a high liquid pressure has been developed and is more practical.[38-42]. So hydrodynamics-based administration could be an efficient, safe and convenient way in the study of gene therapy for diabetes, especially in study of the function of gene products and the practicality of gene expressing vectors.

In conclusion, hydrodynamics-based approach can transfer insulin cDNA efficiently into diabetic mice livers. High level expression of insulin protein can result in significant reduction of blood glucose levels and improve diabetic syndromes. This intravenous procedure could be a convenient and efficient way in study of gene transfer and expression in diabetic mice.

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