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Gene therapy for streptozotocin-induced diabetic mice by electroporational transfer of naked human insulin precursor DNA into skeletal muscle in vivo

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Abstract Transfer of naked plasmid with insulin precursor DNA into skeletal muscle of streptozotocin (STZ)-induced diabetic mice through electroporation and detection of gene expression is described. Four different human insulin precursor DNA fragments were inserted into pcDNA3.1(−), downstream of a CMV promoter. Three of them, with a secretion signal sequence, succeeded in lowering blood glucose at a range of 30–50% in STZ diabetic mice. The other, with a synthetic DNA fragment encoding human proinsulin, failed. The mortality rate of very seriously STZ diabetic mice was reduced significantly by the treatment. The circulating insulin-like protein (mouse insulin, human proinsulin, or intermediates during conversion of proinsulin to insulin) level in the blood of less seriously STZ diabetic mice treated with the human preproinsulin gene with an intron was about 15–23 μU/ml, while that of STZ diabetic mice treated with empty vector was only about 6 μU/ml and that of normal mice was about 18 μU/ml. Transcription of the three human insulin precursor DNAs in mouse skeletal muscle was also detected by RT-PCR. The human preproinsulin gene with the intron showed a slightly higher potency in reducing blood glucose of mildly diabetic mice. These studies indicate that the skeletal muscle transferred with appropriate preproinsulin DNA by electroporation in vivo can secrete insulin-like protein resulting in reduction of blood glucose, and a basal blood insulin level can be achieved for at least 1 month.

Key words: Gene therapy; Diabetic mouse; Insulin precursor DNA; Electroporation; Skeletal muscle

1. Introduction

In most cases, genes are transferred by infectivity of viral particles in gene therapy. Retroviruses can achieve efficient integration, but with the disadvantages of low titer, instability of the viral vector and requirement for target cells in division. Adenoviruses can provide more efficient gene transfer, but with limitations such as the risk of transgene dissemination outside the tumor [1]. Lipofection with cationic liposomes associated with anionic plasmid DNA has been shown to deliver foreign genes efficiently into cells both in vitro and in vivo [2,3]. However, endocytosed plasmid DNA is liable to be digested in the lysosomes, and cationic liposomes have cytotoxicity [4]. Among the methods of gene transfer, electroporation can efficiently introduce plasmid into skin [5], chick embryo [6], liver [7], and muscle [8–11]. Intramuscular electrotransfer can strongly increase reporter and therapeutic gene expression by several orders of magnitude as compared to naked DNA [8–10] and the expression can last for up to 9 months [8]. Plasmid gene transfer by electroporation has several advantages over transfer using viral vectors: a large quantity of highly purified plasmid DNA can be easily and inexpensively prepared; plasmid vector can carry larger genes; a mixture of two or more different plasmid constructs can be co-transferred into muscle by electroporation [11]. Plasmid DNA is an unintegrated form, but can express in myofibers [12]. Compared with other gene transfer methods, transferring genes into muscle is simple, safe and inexpensive [8–13], and may have much higher potential in clinical applications.

Insulin-dependent diabetes mellitus (IDDM) is caused by a shortage of insulin secretion. Since insulin-producing β cells are destroyed to a certain degree, gene therapy for IDDM has to find a way to transfer the insulin gene into an ectopic organ. The exocrine glands of the gastrointestinal system, such as the liver, have been frequently used as target organs [14,15]. Bartlett et al. [16,17] proved that skeletal muscle could secrete insulin-like protein after direct injection of naked insulin precursor DNA. In our investigation, to undertake a simple approach to gene therapy for diabetes and to compare the treatment effects of different insulin precursor DNA constructs, skeletal muscle was selected as the target organ. Four expression plasmids with different insulin precursor DNAs were constructed and transferred into skeletal muscle of streptozotocin (STZ) diabetic mice by electroporation in vivo. The effects of gene therapy on STZ diabetic mice are reported.

2. Materials and methods

2.1. Materials

The human preproinsulin gene in pBAT16.hinsG1.M2 was kindly provided by Dr. Michael S. German, University of California at San Francisco, CA, USA [18]. There is a furin recognition site mutation between the C peptide and the A chain. The gene has an intron within the sequence of the C peptide and is 1119 bp large. The human preproinsulin cDNA in pT7hPPI-1 was kindly provided by Dr. Delphine Mitanchez, Institut Cochin de Génétique Moléculaire, Paris, France (originally provided by Dr. Kevin Docherty, Department of Molecular and Cell Biology, Aberdeen, UK). The pBCA containing synthetic DNA encoding human proinsulin was a gift from Dr. Tongjian Shen of the Institute of Biophysics, Academia Sinica (originally provided by Dr. Ray Wu [19]). Expression vector pcDNA3.1(−) was purchased from Invitrogen. Restriction enzymes, T4 DNA ligase, all

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components required for production of cDNA and RT-PCR analysis were purchased from Promega. STZ was purchased from Sigma.

2.2. Construction of a mutant human preproinsulin cDNA

The human native preproinsulin cDNA in pT7hPPI-1 was mutated by PCR to make a furin recognition site between the C peptide and the A chain. The 5′-primer IN5, 5′-ccagaattcatggccctgtggatg-3′, and mutant primer INBC, 5′-tctgccgggatccctccagggc-3′, were used to get the upstream fragment. The complementary mutant primer INA, 5′-gagggatcccggcagaagcgtg-3′, and 3′-primer IN3, 5′-actaagcttctagttg-cagtagttctccag-3′, were used to get the downstream fragment. The mutant preproinsulin cDNA was then obtained by another PCR with the above two fragments as template and with primers IN5 and IN3. The mutant sequence between the C peptide and the A chain is the same as that of the mutant human preproinsulin gene in pBA-T16.hinsG1.M2. The mutation was confirmed by DNA sequencing.

2.3. Construction of the eukaryocyte expression plasmids

All plasmids were constructed by routine molecular cloning techniques [20]. The vector pcDNA3.1(−) contains a CMV promoter. The human preproinsulin gene in pBAT16.hinsG1.M2 was amplified by PCR with primers IN5 and IN3 and introduced into pcDNA3.1(−) to construct pCMV-IN. Likewise, the human preproinsulin cDNA in pT7hPPI-1 was introduced into pcDNA3.1(−) to get pCMV-IN-cDNA and the mutant human proinsulin cDNA was inserted to get pCMV-INcDNAm. All above foreign DNA fragments were inserted into the multi-cloning site of pcDNA3.1(−) between the *Eco*RI and *Hin*dIII sites. The pBCA was digested by *Eco*RI and *Bam*HI and the synthetic human proinsulin DNA was isolated from agarose gel and cloned into the multi-cloning site of pcDNA3.1(−) to get pCMV-proIN. All above human insulin precursor genes are shown in Fig. 1 and controlled by the CMV promoter.

2.4. Treatment of animals

Male C57BL/6J mice (from the medical experimental animal center, Peking University), 6–8 weeks, weight about 18–20 g, were injected intraperitoneally with a dose of 40 mg STZ/kg body weight on five consecutive days. STZ was dissolved in a 10 mmol/l sodium citrate solution with 0.9% NaCl, pH 4.5, immediately before administration. The mice with blood glucose levels in the range of 15–30 mmol/l were selected and used 20–30 days after STZ treatment. All mice were fed at room temperature with sufficient water and food.

2.5. DNA injection and electroporation

100 μg of DNA in 100 μl of 0.9% NaCl was injected into two back sural muscles of each mouse. Transcutaneous electric pulses were applied by two stainless steel needle electrodes placed about 5 mm apart on each side of the muscle just after DNA injection. The delivery of pulse was 40 ms and repeated 10 times. The electric field strength was 200 v/cm, frequency 1 Hz. There were at least five mice in each experimental group.

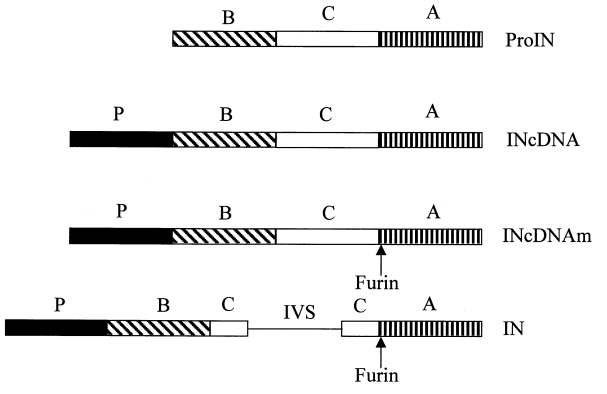

Fig. 1. Structure of four human insulin precursor DNAs. P, secretion signal sequence; B, insulin B chain; C, C peptide; A, insulin A chain; IVS, intervening sequence or intron. There is a furin protease site between the C peptide and the A chain in INcDNAm and IN. Four human insulin precursor DNAs were cloned into pcDNA3.1(−) under the control of a CMV promoter.

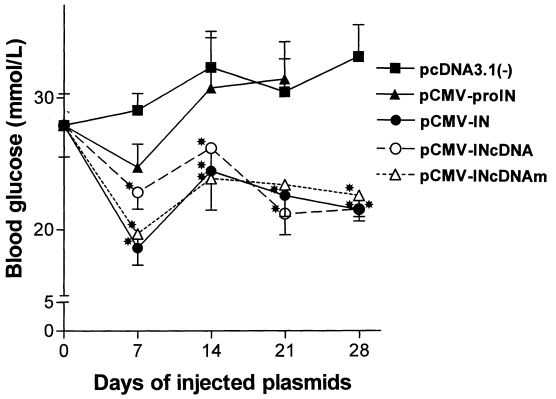

Fig. 2. The effect of various plasmids with different insulin precursor DNAs on the blood glucose of severely diabetic mice. Mice with blood glucose levels of 25–30 mmol/l were selected and used 20–30 days after STZ treatment. Various plasmids were injected. pcDNA3.1(−) was used as a control. 100 μg DNA for each mouse was injected and electroporated. Blood samples from the tail were obtained 7, 14, 21, or 28 days later, and glucose contents were determined on a Glucotrend monitor with Glucotrend glucose test strips. All mice were fed at room temperature with sufficient water and food. Blood glucose levels in healthy non-STZ-treated mice were 5.89 ± 0.29 mmol/l; $n = 5$; $^*P < 0.05$.

2.6. Blood glucose and serum insulin-like protein assays

Blood samples collected from the tail were used to measure blood glucose directly with a Glucotrend monitor and Glucotrend Glucose test strips (Roche Diagnostics, detection range: 0.6–33.3 mmol/l). Blood samples for insulin assay were collected from bulbus oculi, and centrifuged at $8000 \times g$ for 10 min. Serum was kept at −20°C until serum insulin-like protein was measured by RIA. Rabbit polyclonal anti-human insulin antibodies, which have a cross-reactivity of 40–50% to mouse insulin (Navy R.I.A. Technique Center, Beijing), were used in the assay. All samples were collected at 15.00–17.00 h.

2.7. RT-PCR assay of human insulin precursor DNA transcription in severely diabetic mouse muscle

Three weeks after injection of human insulin precursor DNA in severely diabetic mouse muscle, about 100 mg of injected muscle tissue was dissected. Total RNA of the muscle sample was prepared by acid guanidinium thiocyanate–phenol–chloroform extraction and treated with DNase I to eliminate contaminated genomic DNA.

Specific preproinsulin gene primers IN5 and IN3, and β-actin primers 5′-gtggggcgcccaggcacct-3′ and 5′-cttccttaatgtcacgcacgattg-3′ were used for RT-PCR analysis. The PCR was done as 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, 30 cycles, followed by an extension of 8 min at 72°C. The PCR products were analyzed by 1.5% agarose gel electrophoresis. The lengths of the expected PCR products were 340 bp for preproinsulin and 545 bp for β-actin.

2.8. Statistics

Data are given as mean ± S.E.M. Statistical significance of differences between groups was determined by Student's *t*-test. The level of significance is $P < 0.05$.

3. Results

3.1. Reduction of blood glucose in insulin precursor DNA-treated STZ diabetic mice

Human insulin precursor DNA expression constructs under the control of the CMV promoter were prepared by molecular cloning techniques and the gene structures are demonstrated in Fig. 1. To determine the effect of transfer of naked plasmids with insulin precursor DNA into muscle, the blood glucose of STZ diabetic mice was measured. Blood glucose pro-

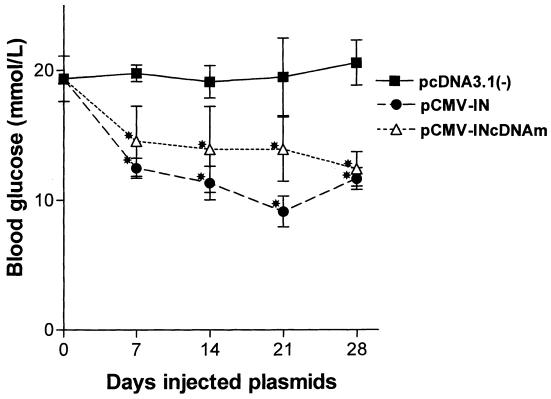

Fig. 3. The effect of various plasmids with insulin precursor DNAs on the blood glucose of mildly diabetic mice. Mice with blood glucose about 20 mmol/l were selected for injection of various plasmids. pcDNA3.1(−) was used as control. 100 μg DNA for each mouse was injected and electroporated. Blood samples from the tail were obtained 7, 14, 21, or 28 days later, and glucose contents were determined on a Glucotrend monitor with Glucotrend glucose test strips. All mice were fed at room temperature with sufficient water and food. Blood glucose levels in healthy non-STZ-treated mice were 5.89 ± 0.29 mmol/l; $n = 6$; $^*P < 0.05$.

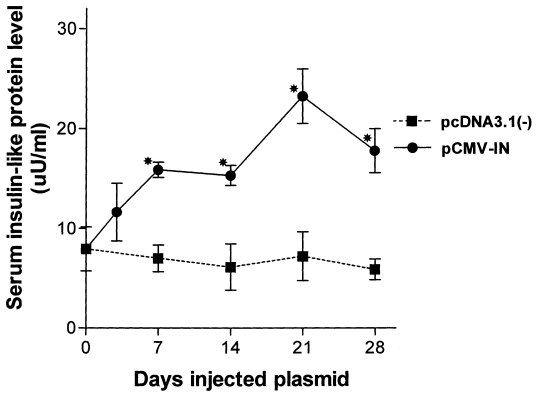

Fig. 4. The effect of pCMV-IN on serum insulin-like protein of mildly diabetic mice. Blood samples for insulin assay were collected from bulbus oculi, and centrifuged at 8000×*g* for 10 min. Serum insulin-like protein (mouse insulin, human proinsulin, and some possible intermediates during conversion from proinsulin to insulin) was measured by RIA with human insulin polyclonal antibodies. The serum insulin of normal mice is 18.0 ± 3.8 μU/ml; $n = 5$; $^*P < 0.05$.

files of 28 days were established at 7 day intervals. The severely diabetic mice with blood glucose levels of 25–30 mmol/l were selected, and four types of plasmids were transferred separately. Three of them, pCMV-IN, pCMV-INcDNA and pCMV-INcDNAm, could reduce about 30% of blood glucose at the most efficient. pCMV-proIN, however, had only a small effect of abating blood glucose (Fig. 2). When the illness of STZ diabetic mice was less serious at about 20 mmol/l blood glucose level, the blood glucose could be reduced to 50% by pCMV-IN (Fig. 3).

3.2. Evaluation of serum insulin-like protein in mildly diabetic mice treated with pCMV-IN

The serum insulin-like protein was measured by RIA with human insulin polyclonal antibodies. Normal mice and STZ diabetic mice transferred with pcDNA3.1(−) were used as controls. The insulin-like protein level in mildly diabetic mice treated with pCMV-IN was much higher (15–23 μU/ml) than with pcDNA3.1(−) (about 6 μU/ml) (Fig. 4), and was nearly equal to that of normal mice (about 18 μU/ml).

3.3. RT-PCR assay of human insulin precursor mRNA in severely diabetic mouse skeletal muscle

Twenty-one days after mice were injected with pCMV-IN, pCMV-INcDNA or pCMV-INcDNAm, the presence of insulin precursor mRNA transcribed in the muscle tissue was determined by RT-PCR. The insulin precursor mRNA was detected in muscle tissue injected with human insulin precursor DNA, but not with empty vector pcDNA3.1(−) (Fig. 5).

3.4. Mortality rate decrease for very seriously STZ diabetic mice with insulin precursor DNA treatment

When mice were treated with STZ at an overdose of 50 mg/kg body weight on five consecutive days, the mice would have a very serious diabetes after 3 weeks, and most of them would die within the next 4 weeks. If injected with either pCMV-IN or pCMV-INcDNA, the mortality rates were clearly decreased (Table 1), but not with pCMV-proIN.

4. Discussion

Muscle as the target tissue for foreign gene transfer has been demonstrated to result in transgene expression and therapeutic responses [8–12,16,17,21,22]. Muscle may be particularly appropriate to take up and express foreign DNA because of its multinucleated cell, sarcoplasmic reticulum, and transverse tubule system, which contains extracellular fluid and penetrates deep into the muscle cell [12]. The DNA may enter damaged muscle cells, which then recover [12]. Muscle is useful in delivering cytokines, growth factors, and other serum proteins by intramuscular DNA injection [11]. That the ectopic insulin-like protein was expressed in skeletal muscle in the present investigation provides another line of evidence.

Gene transfer is often one of the limiting steps in gene therapy. Electroporation provides a more efficient method of gene transfer in vivo [1,7–11]. Electroporation is a technique involving the application of short-duration, high-intensity electric pulses to a cell or tissue [23]. The electrical stimulus causes membrane destabilization and subsequent formation of nanometer-sized pores. In this permeabilized state, the mem-

Table 1
The effect of human insulin precursor DNAs on the mortality rate of very seriously STZ diabetic mice

Plasmid with insulin DNA	Beginning number of STZ diabetic mice	Number of deaths in STZ diabetic mice in 4 weeks	Mortality rate of STZ diabetic mice (%)
pcDNA3.1(−)	28	24	85.7
pCMV-proIN	20	16	80.0
pCMV-IN	19	4	21.1
pCMV-INcDNA	19	5	26.3

The blood glucose of very seriously STZ diabetic mice was over 33 mmol/l.

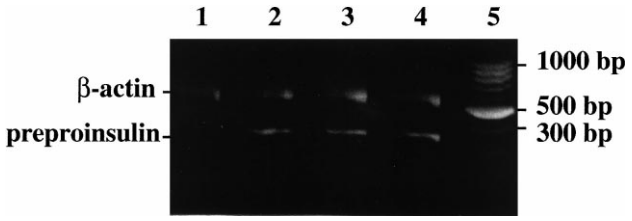

Fig. 5. RT-PCR analysis of human insulin precursor mRNA in severely diabetic mouse muscle injected with various plasmids. 1.5% agarose gel was used to analyze the PCR products of human insulin precursor gene expression. Muscle tissue of about 100 mg was collected 3 weeks after plasmid DNA injection. mRNA samples were prepared and cDNA products were obtained and subjected to PCR. The mRNAs of preproinsulin were detected from muscles injected with pCMV-IN (lane 2), pCMV-INcDNA (lane 3) and pCMV-INcDNAm (lane 4), but not with pcDNA3.1(−) (lane 1). The mRNA of β-actin from the respective tissues was used as a control. Ladder DNA marker is 100 bp grades from 100 to 1000 bp (lane 5). The cDNA length for preproinsulin is 340 bp and and for β-actin 545 bp.

brane can allow passage of macromolecules into the cell [1]. Electroporation has been used to transfer plasmid DNA in vivo to many kinds of organs [5,7–11]. Electroporation was equally efficient in transferring genes to both regenerating and normal muscle [11]. Mir et al. reported an efficient method for DNA transfer into muscle fibers, which consists of plasmid DNA intramuscular injection, followed by delivery of low-field-strength, long-duration, square-wave electric pulses through external or invasive electrodes [8]. The expression of *Photinus pyralis* peroxisomal luciferase could last for more than 9 months in mice [8]. A similar method was used in our study.

Four different human insulin precursor DNAs were used in our study. Among these insulin precursor DNAs, the synthetic DNA encoding human proinsulin without signal peptide in pCMV-proIN could not efficiently reduce the blood glucose of STZ diabetic mice (Fig. 2). pCMV-proIN also showed no effect on the reduction of the mortality rate of very seriously diabetic mice as compared with pCMV-IN and pCMV-INcDNA, which have the signal peptide (Table 1). This indicates that the signal peptide is probably necessary for the insulin precursor to be successfully secreted from the muscle cells. pCMV-IN with the human preproinsulin gene could reduce the blood glucose of mildly diabetic mice more efficiently than pCMV-INcDNAm with human preproinsulin cDNA (Fig. 3). The difference between the human preproinsulin gene and human preproinsulin cDNA is that there is an intron in the preproinsulin gene. The intron may enhance the transcription of the preproinsulin gene in the muscle cell. This suggests that using preproinsulin genomic DNA may be better than using preproinsulin cDNA in gene therapy for diabetes.

In pancreatic β cells, proinsulin is processed into mature insulin by proteases, PC2 and PC3 [24]. PC3 cleaves at the Arg-Arg sequence in the B chain and C peptide junction. PC2 cleaves at the Lys-Arg sequence in the C peptide and A chain junction [25]. Normally this maturation process from native proinsulin does not occur in any non-endocrine cell through the lack of the specific proconvertases. One possibility to overcome this limitation in non-endocrine cells is to engineer some specific sequence for processing. The sequence at the AC junction can be changed to give a site that can be cleaved by furin, a ubiquitous endoprotease [26], to allow proinsulin being processed to mature insulin in non-endocrine cells [14,27]. The Arg-X-Lys/Arg-Arg sequence is a signal for furin cleavage. In our study, the human preproinsulin gene in pCMV-IN has a native sequence Lys-X-Arg-Arg for the BC junction and a mutant sequence Arg-X-Lys-Arg for the AC junction. Human insulin was reported to have almost equal potency in stimulation of glucose oxidation activity as compared with mouse insulin in the rat adipocyte assay, while proinsulin only showed 2–3% of insulin activity [28]. The blood glucose of transgenic STZ diabetic mice did show a decrease (Figs. 2 and 3), but never dropped to the normal level in spite of their nearly normal serum insulin-like protein level (Fig. 4). The pCMV-INcDNA construct gave a similar potency in reducing blood glucose as both pCMV-IN and pCMV-INcDNAm with the furin cleaving site (Fig. 2). This indicates that the proinsulin expressed in muscle cells was not efficiently processed into highly active insulin. The furin may be not abundant in skeletal muscle cells. The serum insulin-like protein we detected may include mouse insulin, human proinsulin, and possibly some intermediates during conversion of proinsulin to insulin. Successful transcription of the preproinsulin genes in muscle tissues has also been identified by RT-PCR analysis (Fig. 5).

The data analysis for glucose level reduction, mortality rate reduction, secretion of insulin-like protein and RT-PCR results indicates that the skeletal muscle is an excellent target organ for ectopic insulin precursor gene expression. Although we are using constitutive secretion to produce insulin-like protein rather than regulated secretion, none of the mice with sufficient water and food suffered from hypoglycemia in our experiment. The Diabetes Control and Complications Trial suggested that tight control of glycemia could prevent or appreciably reduce the incidence of long-term complications associated with IDDM [29]. However, relatively few patients are capable of complying with the strict regimens required for achieving euglycemia with open loop insulin treatment [13]. Using muscle as a target organ for gene therapy of diabetes may protect and reduce complications by keeping a basal insulin level in patients. It may be relatively a safe and simple approach of gene therapy for diabetes. Our data also indicate that the transgenic muscle tissue can keep expressing insulin-like protein for more than 1 month. This is especially good as patients would receive only one injection in more than 1 month instead of three daily injections of insulin protein.

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