Direct transfer of A20 gene into pancreas protected mice from streptozotocin-induced diabetes[1]

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KEY WORDS adenoviridae; streptozocin; insulin-dependent diabetes mellitus; gene therapy; nitric oxide; glucose; amylases; reversed transcriptase polymerase chain reaction; Western blotting

ABSTRACT

AIM: To investigate the efficiency of transfer of A20 gene into pancreas against STZ-induced diabetes. **METHODS:** PVP-plasmid mixture was directly transferred into the pancreatic parenchyma 2 d before STZ injection. The uptake of plasmid pcDNA3-LacZ or pcDNA3-A20 was detected by PCR and the expression of LacZ was confirmed by histological analysis with X-gal. A20 expression in the pancreas of pcDNA3-A20 transgenic mice was measured by RT-PCR and Western blots. Urine amylase, NO generation, and histological examination were examined. **RESULTS:** Injection of PVP-plasmid mixture directly into the pancreatic parenchyma increased urine amylase concentration 16 h after operation and reversed it to nearly normal 36 h later. On d 33 LacZ expression could be found in spleen, duodenum, and islets. The development of diabetes was prevented by direct A20 gene transferring into the pancreas and A20-mediated protection was correlated with suppression of NO production. The insulitis was ameliorated in A20-treated mice. **CONCLUSION:** Injection of PVP-plasmid mixture directly into the pancreatic parenchyma led to target gene expression in islets. Direct transfer of A20 gene into the pancreas protected mice from STZ-induced diabetes.

INTRODUCTION

Type I diabetes mellitus is a T-cell-dependent autoimmune disease resulting in selective destruction of the β cells of the islets of langerhans. β Cell apoptosis has been associated with IDDM onset in both animal models and newly diagnosed diabetic patients[1,2].

The multiple low-dose streptozotocin (STZ) mouse model mimics, in some basic aspects, recent onset of type I diabetes in human patients. Injection of STZ in 5 equal low doses (45 $mg \cdot kg^{-1} \cdot d^{-1}$) induces a slow progressive hyperglycemia, accompanied by lymphocytic infiltration of the pancreatic islets[3]. Apoptosis was the way of cell death responsible for loss of β cells in this STZ model and apoptosis was also correlated with the onset of both insulitis and diabetes[4]. This method was used as a good model to investigate the type I diabetes mellitus.

A20 was originally described as an anti-apoptotic gene induced by TNF-α in endothelial cells[5,6]. Besides the protective effects against apoptosis, A20 also has the protective effects on proinflammatory responses in

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endothelial cells[7,8]. Recombinant adenovirus (rAd)-mediated gene expression of A20 in rodent islets protected against cytokine-induced apoptosis and inhibited cytokine-induced NO generation[9].

Gene transfer into the rat ductal epithelium, acinar cells, and islets of Langerhans was accomplished with recombinant adenovirus by retrograde delivery of adenovirus into the pancreaticobiliary duct[10-12]. However, the adenoviral transgenic expression in the pancreas was transient, because all gene expression was lost by d 28 with an associated lymphocytosis noted at the sites of viral transduction[13]. Besides the potentially higher biological risk of virus gene, the antigenicity of the vector also presented a formidable obstacle[14]. Direct injection of recombinant adenovirus into the pancreas led to the production of neutralizing antibodies and sensitized splenocytes which engaged in increased cytotoxic, lymphoproliferative, and cytokine release activity when reexposed to adenovirus. However, polyvinylpyrrolidone (PVP)-plasmid was a nonviral, polymeric interactive non-condensing gene delivery system[15]. So PVP system was selected to transfer A20 gene into pancreas. The present study aimed to observe the protective effects of A20 gene against STZ-induced diabetes.

MATERIALS AND METHODS

Recombinant vectors A20 gene was cloned from TNF-α- and CHX-induced human umbilical vein endothelial cell (HUVEC) line by RT-PCR. Human A20 gene was cloned into pcDNA3 with the restricted sites *Kpn*I and *Xba*I, which was named pcDNA3-A20. pcDNA3-LacZ was used as a marker. DNA was purified with Qiagen Midi Column to assure that no endotoxin was involved. Before gene transfer, 100 μg DNA was mixed with 5 % PVP and put at room temperature (22-25 ºC) for 15 min, then osmotic pressure (OP) was adjusted with 0.9 % normal saline. Total volume was 120 μL.

Diabetes C57BL/6J male mice between 6-8 weeks of age were used in all experiments. Animals were maintained in standard environmental conditions with free access to food and water. They were allowed to adapt to their environment for 1 week before initiating the experiments. On d 0, following an intraperitoneal (ip) injection of sodepent 40 mg/kg, a laparotomy was performed on all animals and the distal pancreas were identified. Using a 33-gauge needle, 120 μL of a recombinant vectors (100 μg pcDNA3-A20, 100 μg pcDNA3-LacZ, or 100 μg pcDNA3) suspension was injected directly into the pancreatic parenchyma. Two days later (d 2), mice were injected ip STZ 45 mg/kg for 5 daily doses (streptozotocin, Sigma) which was dissolved in sodium citrate buffer (pH 4.5) just before use[3].

The onset of diabetes was determined by measurement of the glucose concentration in blood obtained from a tail vain by blood glucose test strips (Glucotrend Plus, Roche). Consecutive readings of blood glucose levels above 13.9 mmol/L were considered diagnostic of diabetes onset. It was measured at least twice a week.

Amylase activity Urine samples were obtained at 16 h, 36 h, and 72 h after gene transfer. Urine amylase was determined with classical iodine colorimetry[16]. Amylum was used as the substrate.

Analysis of LacZ gene transfer by polymerase chain reaction (PCR) Genomic DNA was prepared from pcDNA3 or pcDNA3-LacZ transgenic tissues using standard Proteinase K (Merck) digestion and phenol/chloroform extraction conductions. Primers were as following: P1: 5'-GCC AGC GCG GAT CAT CGG TCA GAC G-3'; P2: 5'-GCC TGC GAT GTC GGT TTC CGC GAG G-3'. PCR was performed using 100 ng of genomic DNA for 35 cycles of denaturing (94 ºC, 1 min), annealing (60 ºC, 2 min), and polymerization (72 ºC, 2 min).

Histological analysis with X-gal Mice were killed at d 33. Tissues from mice injected with pcDNA3-LacZ were fixed in 4 % PFA at 4 ºC overnight and embedded in OCT and frozen in liquid nitrogen for the freeze-sections. The slides were rinsed with PBS containing $MgCl_2$ 1 mmol/L for 10 min and then placed in X-gal solution [0.5 g/L X-gal, $K_3Fe(CN)_6$ 5 mmol/L, $K_4Fe(CN)_6$ 5 mmol/L, and $MgCl_2$ 1 mmol/L in PBS, pH 7.4] and incubated at 37 ºC for 3-6 h. The slides were counterstained for 20 min with hematoxylin.

Analysis of A20 gene transfer by reverse transcriptase (RT)-PCR Whole RNA of pcDNA3-A20 or pcDNA3 transgenic pancreas was extracted with Trizol Reagent (GIBCO) according to the manufacturer's instructions, and then identified by electrophoresis. Random hexamer primers (Promege) were employed for cDNA preparation using the MMLV reverse transcriptase (Promega). PCR was performed with as following: denaturing at 95 ºC for 1 min, annealing at 62 ºC for 1.5 min, and synthesis at 72 ºC for 2.5 min (Last cycle for 10 min), totals for 35 cycles. For detection of A20, the primers used were as following: for-

ward primer: 5'-CGG TAC CGC ACA ATG GCT GAA CAA GTC CTT CCT C-3', reverse primer: 5'-CGT CTA GAG TTA GCC ATA CAT CTG CTT GAA CTG-3'. Primers for housekeeping gene β-actin were as following: forward primer: 5'-AAC GAG CGG TTC CGA TGC CCT GAG-3', reverse primer: 5'-TGT CGC CTT CAC CGT TCC AGT T-3'. Amplification products (20 %) were separated by electrophoresis on a 1.5 % agrose gel and visualized by ethidium bromide staining.

Analysis of A20 expression with Western blots The pancreas were smashed after incubation in liquid nitrogen, suspended in suspending buffer (NaCl 0.1 mol/L, Tris-HCl 0.01 mol/L, pH 7.6, egtazic acid 1 mmol/L, aprotinin 1 mg/L, and PMSF 100 mg/L), immediately 2×SDS loading buffer was added with equal volume, and the mixture was boiled for 10 min. After centrifugation, the lysates were sonicated for 10 s and incubated on ice for 10 min. Sample 30 μL was resolved on 10 % SDS-PAGE. Proteins were electrotransferred onto a nitrocellulose membrane. The membranes were blocked with 5 % non-fat milk and probed with mouse anti-hA20 antibody (Oncogene). The blots were washed and exposed to HRP-conjugated anti-mouse IgG secondary antibody (Santa Cruz, CA) and then developed using the ECL reagent. The molecular mass of the protein was estimated relative to pre-stained size marker (Bio-Rad).

Determination of NO generation in diabetic pancreas The pancreas was smashed after incubation in liquid nitrogen, suspended in suspending buffer (NaCl 0.1 mol/L, Tris-HCl 0.01 mol/L, pH 7.6, egtazic acid 1 mmol/L, aprotinin 1 mg/L, and PMSF 100 mg/L). After centrifugation, 50 μL Griess reagent (equal volume of 1 % sulffanilamide in HCl 0.1 mol/L and 0.1 % *N*-[-1-naphthyl-ethylenediamine dihydrochloride]) was added to 50 μL of suspending media. Nitrite concentration was determined by spectrophotometry (560 nm) from a standard curve (0-100 mmol/L) derived from $NaNO_2$ (Beyotime Biotechnology). NO data was expressed as mean±SD (nitrite) in μmol/L.

Histological examination of diabetic pancreata Mice were sacrificed at d 33. The pancreata were fixed in 4 % paraformaldehyde in PBS 0.01 mol/L (pH 7.4), processed for paraffin embedding, sectioned (6 μm), and stained with hematoxylin-eosin.

To determine the severity of insulitis, more than 30 pancreatic islets from three or more parallel sections of different cut levels were analyzed per mouse. The degree of insulitis was classified into four categories: 0, no insulitis; 1, periinsulitis with or without minimal lymphocyte infiltration in islet; 2, invasive insulitis with islet destruction ≤50 %; 3, islet destruction >50 %[17].

Statistical analysis Mean values and difference were analyzed by *t*-test. The *t*-test assuming two samples was performed with the Microsoft EXCEL 2000 data analysis program. A two-tailed *P* value is represented.

RESULTS

Amylase activity Urine amylase was determined as a marker of the damage of exocrine gland. Injection of PVP-plasmid mixture into the pancreatic parenchyma yielded a great increase in urine amylase concentration at 16 h after operation. The urine amylase decreased to nearly normal level at 36 h (Fig 1).

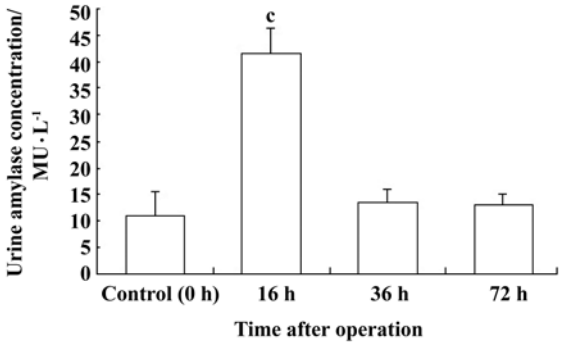

Fig 1. Effect of intra-pancreas injection of 100 μg DNA mixed with PVP on urine amylase activity. *n*=5. Mean±SD. $^cP<0.01$ *vs* control.

Detection of delivered DNA An apparent and specific 318-bp fragment was detected in pancreas, liver, spleen, and duodenum, and a slight fragment was found in stomach and heart (Fig 2).

Histological analysis with X-gal On d 33, positive blue stain was found a lot in spleen and a little in duodenum. But it was variable in islets. However, no acinar cell was found positive (Fig 3). No difference in staining intensity was found between d 5 and d 33 after gene transfer (data not shown).

A20 was expressed in pancreas after gene transfer A specific 2370-bp fragment was found in pcDNA3-A20 transgenic pancreas (Fig 4A). The A20 expression in the pancreas of pcDNA3-A20 transgenic mice was also confirmed by Western blots with a specific 78-kDa fragment (Fig 4B).

Direct A20 gene transfer into the pancreas prevented development of diabetes The blood glucose

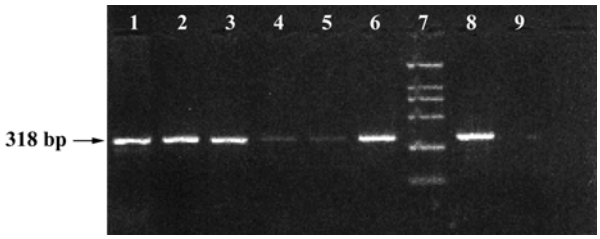

Fig 2. The uptake of plasmid pcDNA3-LacZ was confirmed by PCR. Animals were killed at d 33 after gene transfer. An apparent and specific 318 bp fragment was detected in pancreas and tissues near pancreas such as liver, spleen, duodenum and a slight fragment was found in stomach and heart. Lane1: liver; Lane 2: spleen; Lane 3: duodenum; Lane 4: stomach; Lane 5: heart; Lane 6: pancreas; Lane 7: marker DL-2000; Lane 8: positive control pcDNA3-LacZ; Lane 9: negative control.

level of pcDNA3-A20 transgenic mice was increased more slowly. Until d 24, the difference was significant *vs* control pcDNA3 transgenic mice ($P<0.05$, $n=5$, Fig 5). On d 33, average blood glucose levels of pcDNA3-A20 transgenic mice were lower than 13.9 mmol/L.

NO production There was higher level of NO in the pancreas of pcDNA3 transgenic mice *vs* pcDNA3-A20 transgenic mice killed on d 33 ($P<0.05$, $n=5$). The difference between NO production in pcDNA3-A20 transgenic mice and normal mice was not significant ($P>0.05$, $n=5$, Fig 6).

Morphological examination Mice receiving STZ plus pcDNA3 plasmid revealed apparent insulitis and structural changes of the islets on d 33. A similar histological pattern around the islets was also found in the STZ plus pcDNA3-A20 plasmid-treated mice, but the inflammatory lesions and the degree of insulitis was markedly decreased (Fig 7).

DISCUSSION

The pancreas was an ideal site for potential strategies using gene therapy, because diseases such as diabetes, cystic fibrosis, and pancreatic cancer lent themselves to correction or palliation by specific gene products. Our study showed that a direct transferring PVP-plasmid mixture into pancreas could lead to a stable expression of target gene for at least 33 d. Furthermore, no difference in staining intensity was found between d 5 and d 33 after gene transfer. It meant that this transfer achieved a long-term expression. In addition, gene expression could be found in other tissues indicating that PVP gene delivery system was widely applicable.

Mice treated with STZ did not die after direct transfer of PVP-plasmid mixture into pancreas. Urine amylase was determined as a marker of the damage of pancreas. The increase of urine amylase at 16 h showed that direct injection of PVP-plasmid mixture caused the

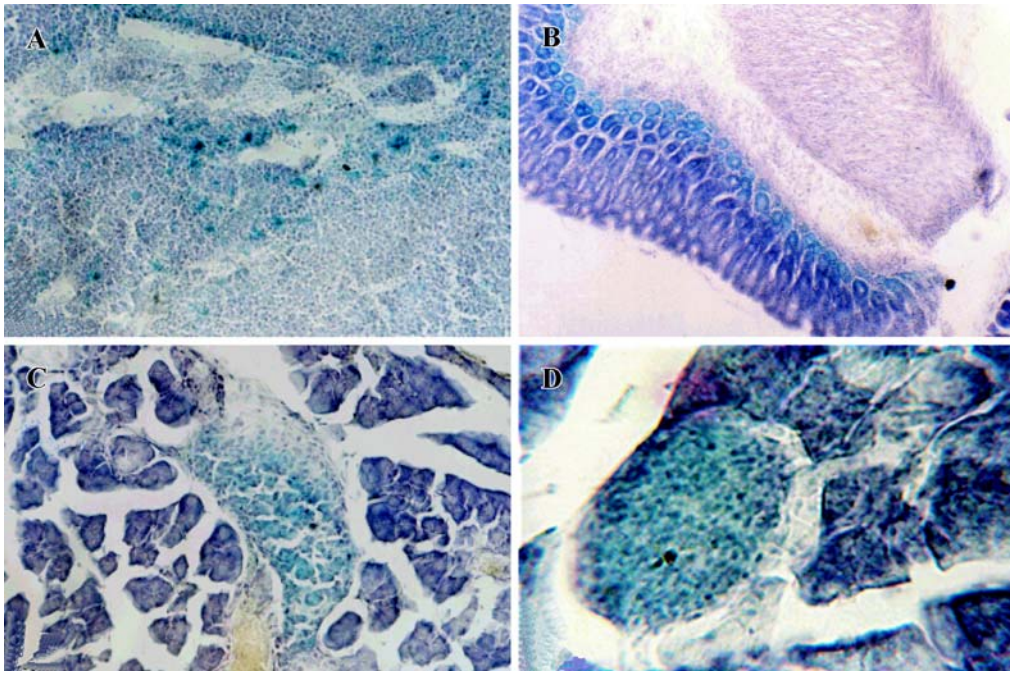

Fig 3. Histological analysis of pancreatic parenchyma after injection of pcDNA3-LacZ mixed with PVP. A: spleen section; B: duodenum section; C and D: pancreas section (×300).

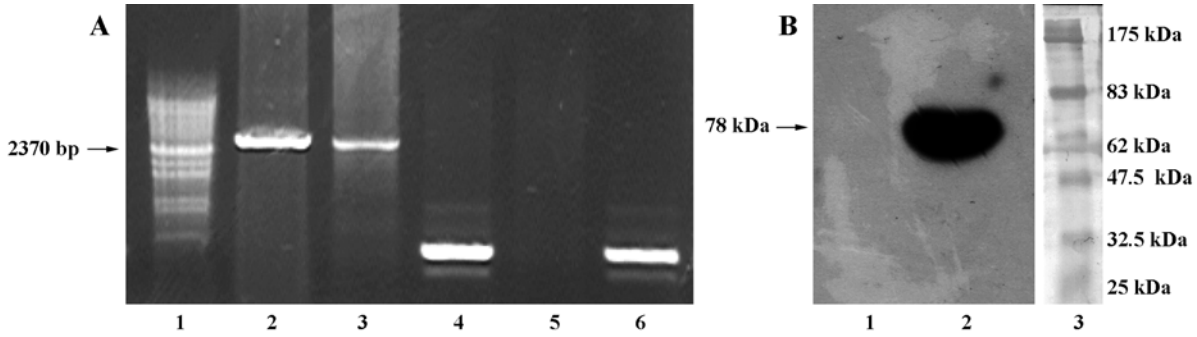

Fig 4. A20 protein expression by Western blotting in STZ-induced diabetes mice after injection of pcDNA3-A20 mixed with PVP. A: A specific 2370 fragment was found in pcDNA3-A20 transgenic pancreas. Lane 1: DNA marker λ/HE; Lane 2: positive control pcDNA3-A20; Lane 3: A20 in the pancreas of pcDNA3-A20 transgenic mice (2370 bp); Lane 4: β-actin in the pancreas of pcDNA3-A20 transgenic mice (591 bp); Lane 5: A20 in the pancreas of pcDNA3 transgenic mice; Lane 6: β-actin in the pancreas of pcDNA3 transgenic mice. B: A specific 78 kDa fragment was found in pcDNA3-A20 transgenic pancreas. Lane 1: A20 in the pancreas of pcDNA3 transgenic mice; Lane 2: A20 in the pancreas of pcDNA3-A20 transgenic mice (78 kDa); Lane3: Protein marker (Bio Rad).

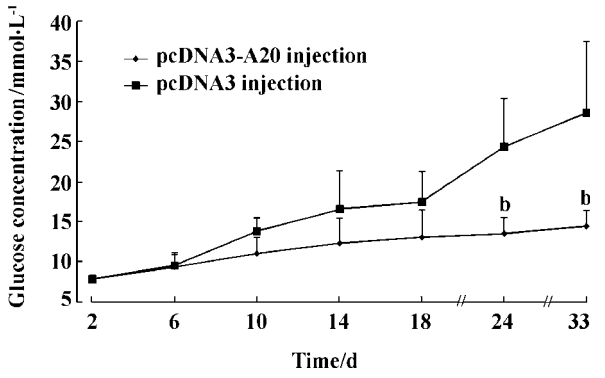

Fig 5. Effects of direct A20 gene transfer into the pancreas on blood glucose level of STZ-induced diabetes mice. Mice were considered diabetes when blood glucose levels were above 13.9 mmol/L. *n*=5. Mean±SD. $^{b}P<0.05$ *vs* control pcDNA3 transgenic mice.

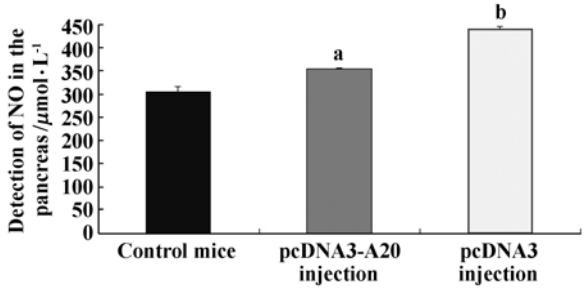

Fig 6. Effect of A20 gene transfer on NO production of STZ-induced diabetes mice. *n*=5. $^{a}P>0.05$, $^{b}P<0.05$ *vs* control.

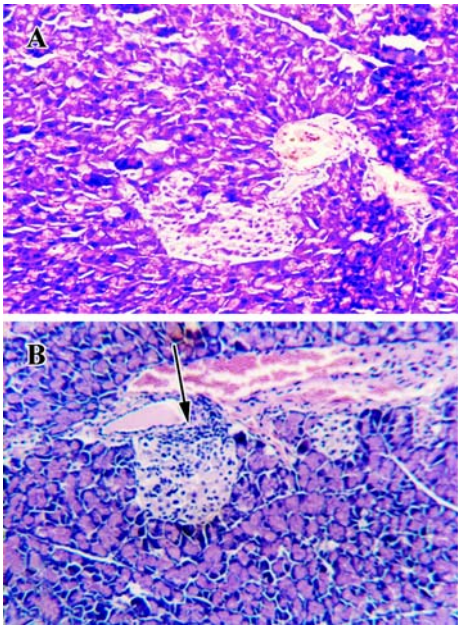

Fig 7. Histological examination of pancreas of pcDNA3-treated and pcDNA3-A20-treated diabetic mice induced by STZ. (A) Example of an islet from a pcDNA3-treated mouse showed severely destroyed islet. Arrow pointed the damage of the islet. (B) Example of an islet from a pcDNA3-A20-treated mouse that displayed light evidence of islet infiltration. Arrow pointed the infiltrated lymphocytes.

impairment in the exocrine gland. However, it could be reversed to nearly normal 36 h later. Therefore the impairment was temporary and resumable. The pancreas of mice were so small and the pancreaticobiliary duct was much smaller, so we injected PVP-plasmid mixture directly into the pancreatic parenchyma. With regard to humans, nonsurgical methods of recombinant plasmid delivery, such as endoscopic retrograde cholangiopancreatography, could serve for gene delivery[13].

A20 suppressed cytokine-induced NO generation

at the level of iNOS transcription through blockade of the transcription factor, nuclear factor κB (NF-κB). It could protect β cells from IL-1β-induced apoptosis[9]. There were substantial evidences that free radical generation, such as release of NO, mediated the progression of type I diabetes[18-20]. Our results showed that STZ-induced diabetes was ameliorated with the suppression of NO.

In summary, direct A20 gene transfer into the pancreas by PVP system could protect mice from STZ-induced diabetes.

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