Genetically engineered K cells provide sufficient insulin to correct hyperglycemia in a nude murine model

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A gene therapy-based treatment of type 1 diabetes mellitus requires the development of a surrogate β cell that can synthesize and secrete functionally active insulin in response to physiologically relevant changes in ambient glucose levels. In this study, the murine enteroendocrine cell line STC-1 was genetically modified by stable transfection. Two clone cells were selected (STC-1-2 and STC-1-14) that secreted the highest levels of insulin among the 22 clones expressing insulin from 0 to 157.2 µIU/ml/106 cells/d. After glucose concentration in the culture medium was increased from 1 mM to 10 mM, secreted insulin rose from 40.3±0.8 to 56.3±3.2 µIU/ml (STC-1-2), and from 10.8±0.8 to 23.6±2.3 µIU/ml (STC-1-14). After STC-1-14 cells were implanted into diabetic nude mice, their blood glucose levels were reduced to normal. Body weight loss was also ameliorated. Our data suggested that genetically engineered K cells secrete active insulin in a glucose-regulated manner, and in vivo study showed that hyperglycemia could be reversed by implantation of the cells, suggesting that the use of genetically engineered K cells to express human insulin might provide a glucose-regulated approach to treat diabetic hyperglycemia.

Keywords type 1 diabetes mellitus; insulin; gene therapy; glucose-dependent insulinotropic polypeptide; STC-1 cell; glucose
constructed, and a tumor-derived K cell line STC-1 [6] was stably transfected with the plasmid. The novel insulin/GIP co-producing cell line was selected. In vitro results suggested that the release of human insulin is glucose-dependent. We also attempted to correct hyperglycemia in vivo in an immunodeficient murine model of diabetes. Blood glucose levels were efficiently lowered by transplantation of insulin-producing STC-1 cells.

Materials and Methods

Construction of plasmids
Plasmid pSK-GIP-hIns was kindly provided by Dr. Burton M. Wice [7] (Washington University School of Medicine, Washington, USA). pcDNA3 was digested with BglII and HindIII, followed by treatment with Klenow polymerase to blunt the ends and self-ligate, resulting in pcDNA3-no-CMV. pSK-GIP-hIns was digested with KpnI and NotI, and the 3 kb fragment of GIP-hIns was isolated and cloned into KpnI and NotI sites of pcDNA3-no-CMV. This resulted in the pcDNA3-GIP-hIns construction used for further studies.

Cells, cell cultures, and generation of stably-transfected STC-1 cells
The murine enteroendocrine cell line STC-1 was obtained from the Institute of Genetics, Fudan University (Shanghai, China). Human normal liver cell line LO2 and normal human lung fibroblast cell line NHLF-1 were purchased from Shanghai Cell Collection (Chinese Academy of Sciences, Shanghai, China). The STC-1 and LO2 cells were cultured in Dulbecco’s minimal essential medium containing 10% fetal bovine serum (Gibco BRL, Gaithersburg, USA), 4 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, Carlsbad, USA). pcDNA3-GIP-hIns was digested with BglII and HindIII, and the 3 kb fragment of GIP-hIns was isolated and cloned into KpnI and NotI sites of pcDNA3-no-CMV. This resulted in the pcDNA3-GIP-hIns construction used for further studies.

RT-PCR analysis
Total cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer’s protocol. RT-PCR was carried out with total RNA (2 µg) using the First strand RT-PCR kit (Stratagene, La Jolla, USA). A cDNA equivalent of 1 ng RNA was amplified by PCR using primers specific for the target genes. The thermal cycles were 94 ºC for 30 s, 58 ºC for 30 s, and 72 ºC for 30 s for 30 cycles for human insulin cDNA (363 bp). Nucleotide sequences of human insulin primers were as follows: sense, 5’-TAGAACCTGGGA-GGGCTAGG-3’; antisense, 5’-CTGTGCCGTCTGT-GTGTTTT-3’. The amplification products were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

Fluorescence microscopy of insulin/pro-insulin staining
Cells were cultured on glass slides. Cells were rinsed once with phosphate-buffered saline (PBS), treated with sucrose buffer and 0.5% Triton X-100 for 2 min on ice, then fixed with 4% paraformaldehyde and rinsed once with 0.5% NP-40 for 5 min. They were incubated with mouse anti-human insulin monoclonal antibody (1:100) (Zymed, South San Francisco, USA) at 4 ºC overnight, followed by incubation with horse anti-mouse secondary antibody (Gibco BRL) labeled with rhodamine for 30 min. Cells were imaged by an Olympus IX70 fluorescence microscope (Olympus, Tokyo, Japan), and the exposure time was set as 200 ms.

Animal care and STC-1 cell implantation
Male BALB/c nude mice at 4–5 weeks old were obtained from the Shanghai Slaccas Animal Laboratory (Shanghai, China). The animals were kept under standard pathogen-free conditions and were handled according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” by the Chinese Academy of Sciences. Animal welfare and all experiments were carried out in accordance with the local ethics committee. The animals were allowed to adapt to the environment 1 week before the start of experiments. Diabetes was induced by intraperitoneal injection of STZ (Sigma), which is dissolved in 100 mM sodium citrate solution (pH 4.5) containing 150 mM NaCl immediately before injection, at the dose of 200 mg/kg body weight to nude mice fasted for 24 h. Mice with blood glucose levels higher than 16.7 mM 48 h after injection were used as recipients [8–10]. Those mice were divided into three groups: group 1 (cell therapy diabetic animals, n=7), mice implanted with STC-1-14 cells; group 2 (cell control diabetic animals, n=6), mice implanted with negative control cells; group 3 (PBS-treated diabetic animals, n=6), mice injected subcutaneously with PBS.

Four to six days after induction of diabetes, 1×10^7 STC-
1-14 cells, control cells, or 200 µl PBS were injected subcutaneously into the lower right flank of athymic BALB/c male nude diabetic mice.

**Measurement of glucose, insulin, and body weight**

Blood glucose levels were checked by a portable glucose tester with a detection limit of 0.6 mM (Accu-Chek Advantage II; Roche Diagnostics, Mannheim, Germany). Non-fasting blood glucose was measured, except as otherwise specified under “Results”, between 08:30 and 09:30 am every 3–4 d. Body weight was measured at the same time. Immunoreactive insulin in the culture medium was measured by a sensitive human insulin radioimmunoassay (RIA) kit (Dongya Immuno-technology Institute, Chinese PLA General Hospital, Beijing, China) standardized against human insulin, according to the protocol provided by the company. This kit is specially developed for human insulin and has no cross-activity with mouse insulin, but can react with proinsulin partially.

The mice were subjected to 5 h of fasting and injected intraperitoneally with 50% glucose at the dose of 5 g/kg body weight. Blood glucose was measured at 30 min intervals up to 120 min after glucose injection. Serum from two or three mice from each group (normal mice, mice that received PBS, control cells, or the engineered K cells) was mixed to have enough volume for the determination of serum insulin levels.

**Immunohistochemistry**

All animals were killed at the end of the study. Tumors were resected and tissue was fixed and embedded in paraffin. Deparaffinized tumor section specimens were treated with 3% H2O2, blocked with SuperBlock (ScyTek Laboratories, Logan, USA), and reacted with mouse anti-human insulin monoclonal antibody (1:100; Zymed), then reacted with biotinylated goat anti-mouse secondary antibody (Gibco BRL), followed by 30 min incubation in the presence of avidin-biotin complex reagent (BioGenex Laboratories, San Ramon, USA) and 5 min exposure to diaminobenzidine. The slides were counterstained with hematoxylin.

**Statistical analysis**

Data are expressed as the mean±SD. Statistical analysis was carried out using Student’s t-test or paired t-test. *P*<0.05 was considered significant.

**Results**

**Generation of insulin-producing STC-1 cell lines**

The insulin-producing STC-1 cell line would be an important tool that could be used to investigate whether engineering insulin production by STC-1 cells is a feasible gene therapy strategy to treat type 1 diabetes mellitus. STC-1 cells were transfected with pGIP/Ins and subjected to genetic selection cultured in the presence of G418. Twenty-two clones were isolated. The clones of cells were analyzed by RIA to determine whether they could secrete human insulin. As shown in Fig. 1(A), human insulin was not detected in either wild-type STC-1 cells or cells transfected with pcDNA3 (control cells). In contrast, 16 out
of 22 clones of cells transfected with pGIP/Ins secreted human insulin. Clone 2 (STC-1-2) secreted the highest level of insulin (157.2 µIU/ml/10^6 cells/d), and clone 14 (STC-1-14) secreted approximately 50% the amount of insulin as STC-1-2. Both STC-1-2 and STC-1-14 were selected for the following studies.

**Identification of expression of human insulin**

To examine exogenous human insulin expression, STC-1-2 cells, control cells, and wild-type STC-1 cells were cultured in 60 mm dishes for 48–72 h, then harvested and subjected to isolation of RNA for RT-PCR or stained for fluorescence microscopy imaging. As shown in Fig. 1(B), the human insulin mRNA of the STC-1-2 group was detectable compared with the control group and the wild-type STC-1 cell group. Human insulin detection by immunofluorescence confirmed the results from RT-PCR (Fig. 2).

**Cell-specific activity of GIP promoter**

To evaluate activation of the GIP promoter that appears to be restricted to STC-1 cells, STC-1, NHLF-1, and LO2 cells were transfected with pGIP/Ins. As shown in Fig. 3, human insulin was detectable only in the medium of the STC-1 cells, and the other two cell lines scarcely secreted human insulin. These results showed that the GIP promoter was cell-specific and likely to be effective in targeting transgene expression specifically to K cells in vivo.

**Glucose sensitivity of engineered STC-1 cells**

To further show that insulin secretion by STC-1 cells is dependent on glucose concentration, two of the STC-1 cell line clones were assayed for insulin release. We analyzed insulin secretion from the cells in culture containing either 1 mM or 10 mM glucose. When STC-1-2 and STC-1-14 cells were cultured in medium containing 1 mM glucose, the release of insulin was 40.3±0.8 µIU/ml and 10.8±0.8 µIU/ml, respectively, whereas 10 mM glucose increased the secretion of insulin to 56.3±3.2 µIU/ml and 23.6±2.3 µIU/ml, respectively. We observed a 1.4- to 2.2-fold difference after the increase in glucose concentration.

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**Fig. 2** Phase-contrast (A, C) and fluorescence (B, D) micrographs of insulin-producing STC-1-14 cells and cells transfected with pcDNA3. STC-1-14 cells (A, B) and cells transfected with pcDNA3 (C, D) were cultured in 60 mm dishes on glass slides for 48–72 h. Human insulin was assayed by the immunofluorescence technique. Magnification, 200×.
This increase was statistically significant ($P<0.001$). This observation suggested that release of human insulin from these cells was glucose-dependent (Fig. 4).

Reversal of hyperglycemia and body weight in diabetic mice by engineered cells

To assess the feasibility of bioengineered STC-1 cells to correct a diabetic state, insulin-expressing STC-1 cells (clone 14) were implanted into diabetic nude mice. Cells were implanted subcutaneously 7 d after successful induction of a diabetic state (blood glucose concentration $>16.7$ mM) through intraperitoneal injection of STZ (200 mg/kg body weight). Animals treated with control cells or PBS served as negative controls. As shown in Fig. 5(A), control cells or PBS-treated diabetic animals remained highly hyperglycemic. In contrast, in the cell therapy group implanted with STC-1-14 clone, blood glucose levels were gradually reduced 26 d after implantation and remained at nearly normal glycemic levels (4–6 mM) until the end of the study in all mice. In one animal we observed hypoglycemic levels (<2.8 mM). These results suggested that a transplant of insulin-releasing cells could decrease blood glucose levels compatible with long-term survival of the recipients. Significant tumor growth was visible macroscopically during this experiment. Mice were killed 49 d after implantation for removal of STC-1-14 tumors. All animals progressively lost weight following STZ injection. The body weight of the mice implanted with the control cells or PBS decreased during the period of the study. However, the diabetic animals that received cell therapy progressively gained body weight [Fig. 5(B)].

Intraperitoneal glucose tolerance test

Normal nude mice, control cell implanted diabetic animals, PBS-treated, or cell therapy diabetic animals were subjected to an intraperitoneal glucose tolerance test. Control cell or PBS-treated diabetic animals were severely hyperglycemic both before and after the glucose ingestion. In contrast, cell therapy diabetic animals showed initial increases in blood glucose followed by significant decreases between 30 and 60 min after glucose ingestion, as normal control mice did. At 90 min, the cell therapy group had blood glucose levels similar to pre-test values [Fig. 6(A)].

Blood samples from each group were also collected for determination of serum insulin levels. To get enough volume of serum for the measurement, the blood from two or three mice was mixed and subjected to RIA analysis. There was no detectable insulin expression in the mice that received PBS or control cells. In the cell therapy group, serum insulin levels increased from 170.9±22.0 µIU/ml before glucose injection to 828.3±72.3 µIU/ml at 30 min after glucose infusion [Fig. 6(B)]. The expression level decreased sharply to 155.4±19.8 µIU/ml at 120 min after glucose infusion. The kinetics of insulin expression was consistent with that of blood glucose levels. These results indicated that human insulin produced from the implanted cells was sufficient to maintain normal glucose tolerance despite having virtually no pancreatic cells.
Cell therapy for type 1 diabetes

Fig. 5 Effect of insulin gene therapy on blood glucose levels and body weight of diabetic mice

The changes in blood glucose (A) and body weight (B) of diabetic nude mice after implantation of cells or treatment with PBS were recorded. Data were plotted as the mean±SEM. Streptozotocin (STZ) treatment was at day S0 (48 h after STZ treatment) and cells were implanted at day D0 (time of cells implantation). W1, a week after STZ treatment. *P<0.05.

Fig. 6 Glucose tolerance test of normal mice and treated diabetic mice

Cell control diabetic (n=3), PBS-treated diabetic (n=3), normal (n=4), and cell therapy diabetic (n=5) animals were subjected to 5 h of fasting from 08.00 hours and injected intraperitoneally with 50% glucose at the dose of 5 g/kg body weight. Blood glucose (A) and insulin (B) levels were measured at 30 min intervals up to 120 min after glucose infusion.

Histological examination of pancreas of normal nude mice and diabetic nude mice, and implanted tumors

The normal architecture of pancreatic islet is shown in Fig. 7(A). The β cells grew in cords or nests with the acinus surrounding the islet full of cells. The islets of diabetic immunodeficient nude mice were almost destroyed 2 months after injection with STZ, and showed thinner cords. The smaller atrophic cells of destroyed islets showed scant cytoplasm and coarse chromatin nuclei [Fig. 7(B)]. The implanted tumor cells were small and spindle-shaped with moderate to little cytoplasm. The cells were closely packed and intensively stained with round nuclei [Fig. 7(C)].
Expression of insulin protein in tumors of treated diabetic mice
Approximately 2 months after cell transplantation, STZ-treated mice were killed and tumors formed by cell implantation were collected and processed for the detection of human insulin expression. Insulin-positive cells were observed in the tumors formed by STC-1-14 cell implantation. As shown in Fig. 8, a brown-colored precipitate indicates the intracellular presence of insulin and/or proinsulin peptides. In contrast, there was no signal in the tumors after control cell implantation.

Fig. 8 Expression of human insulin protein in implanted tumors of treated diabetic mice At the end of the study, mice were sacrificed and tumor sections were stained with antibodies to human insulin. (A) insulin-producing murine enteroendocrine STC-1-14 cell implantation. (B) control cell implantation. Original magnification, 400×.

Fig. 7 Histology of pancreas of normal nude mice and diabetic nude mice, and implanted tumors At the end of the study, mice were sacrificed and the pancreas and tumor sections were stained for histological analysis. The giant island with rich cells of normal nude mouse (A), the small island of diabetic immunodeficient nude mouse (B), and implanted tumor cells (C) are shown. Original magnification, 200×.
Discussion

In this study, we have shown that K cells could be engineered to produce human insulin efficiently. STC-1 cells, a tumor-derived K cell line, are suitable for stable gene transfer and clonal selection, and tumor xenograft could be established in nude mice. Stable transfection allows both the selection of clones with high levels of expression and long-term expression of the human insulin gene. We have shown that significant amounts of human insulin were secreted to the culture medium of STC-1 cells. We also observed that the secretion of human insulin from these cells was glucose-dependent. Two studies [3,11] have previously reported that glucose stimulates insulin secretion approximately 1.5-fold from GIP-producing cell lines. Ramshur et al [7] reported that glucose had no effect on insulin secretion by GIP/Ins cells, although these cells could produce and store human insulin. We are not sure why Ramshur et al [7] got different results, but the differences could be due to the followings. Glucose, the major GIP "secretagogue", does not act directly on gut K cells. Glucose uptake and metabolism by adjacent enterocytes is required for glucose-stimulated GIP release by K cells [12]. Another reason might be the heterogeneity between different clones of GIP-producing cells.

Our results showed that the GIP promoter used is cell-specific and is likely to be effective in targeting transgene expression specifically to K cells in vivo. The mechanism has been unclear until now. Some researchers have identified that two transcription factors, GATA-4 and ISL-1, are involved in the cell-specific transcriptional regulation of GIP [13]. Jepeal et al advocated that cell-specific expression of GIP was regulated by the transcription factor called pancreas duodenum homeobox-1 [14]. But further studies will be necessary to determine whether these transcription factors are functional and requisite for cell-specific expression of GIP.

We implanted transduced STC-1-14 cells into diabetic nude mice and monitored blood glucose and weight gain before and after surgery. These in vivo data indicated that STC-1-14 cells produced mature insulin that significantly reduced blood glucose and permitted weight gain toward normal levels. Control mice that received non-insulin-expressing cells did not show any decrease in blood glucose. A glucose tolerance test provides a rigorous assessment of ectopic glucose-regulated insulin delivery. Diabetic mice implanted with STC-1-14 cells responded to an intraperitoneal glucose tolerance testing by a marked reduction in blood glucose over the 30–60 min time period after glucose challenge. Previous attempts to prevent glucosuria and lethal consequences of diabetes, such as ketoacidosis, were unable to restore normal glucose tolerance [15,16]. Our data show that insulin production from STC-1-14 cells might correct blood glucose and restore normal glucose tolerance (Fig. 6). Cheung et al obtained similar results by establishing transgenic mice expressing human insulin under control of the GIP promoter [3]. It is very important to ascertain whether removal of the tumor results in the return of diabetes. We are carrying out experiments to study this issue and more research needs to be done to make the transplantation of the engineered K cells a feasible treatment for diabetes.

Based on these results, the use of genetically engineered K cells to express human insulin might provide a glucose-regulated approach to reduce diabetic hyperglycemia. However, we must acknowledge the limitation of the study. STC-1 is a tumor-derived K cell line. Significant tumors were visible macroscopically 3 weeks after implantation into nude mice. In our study, one diabetic mouse died of hypoglycemia. An identical problem was found in previous studies [17–19]. Some studies tried to regulate the secretion of insulin by controlling cell overgrowth [20–22], but these methods were not ideal. The primary solution is to develop an effective therapeutic gene delivery system to intestinal cells in the body. Viral vectors have already been developed that deliver genes to cells of the intestinal tract, including stem cells [23–26]. Qin et al revealed that regulated expression of insulin could be achieved by a retroviral vector [27].

We are fully aware of the limitations of our model because of the use of a tumor cell line to secrete insulin, but this work provides a starting point for the treatment of diabetic hyperglycemia through glucose-dependent expression of insulin. Our results showed that genetically engineered STC-1 cells were able to synthesize, process, and secrete active human insulin. Furthermore, release of human insulin from these cells was glucose-dependent. The use of genetically engineered K cells to express human insulin could provide a glucose-regulated approach to reduce diabetic hyperglycemia. The present work is a basic scientific model to prove the concept and the next step is to see whether this concept works after including a long-term expression vector (Adeno-associated virus or gutless adenovirus) carrying the GIP promoter and necessary transcription factors to express insulin.

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