Transplantation of insulin-producing cells to treat diabetic rats after 90% pancreatectomy

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AIM: To investigate the effects of transplantation of insulin-producing cells (IPCs) in the treatment of diabetic rats after 90% pancreatectomy.

METHODS: Human umbilical cord mesenchymal stem cells (UCMSCs) were isolated and induced into IPCs using differentiation medium. Differentiated cells were examined by dithizone (DTZ) staining, reverse transcription-polymerase chain reaction (RT-PCR), and real-time RT-PCR. C-peptide release, both spontaneously and after glucose challenge, was measured by ELISA. IPCs were then transplanted into Sprague-Dawley rats after 90% pancreatectomy and blood glucose levels and body weight were measured.

RESULTS: The differentiated cells were positive for DTZ staining and expressed pancreatic β-cell related genes. C-peptide release by the differentiated cells increased after glucose challenge (380.6 ± 15.32 pmol/L vs 272.4 ± 15.32 pmol/L, P < 0.05). Further, in the cell transplantation group, blood sugar levels were significantly lower than in the sham group 2 wk after transplantation (18.7 ± 2.5 mmol/L vs 25.8 ± 1.25 mmol/L, P < 0.05). Glucose tolerance tests showed that 45 min after intraperitoneal glucose injection, blood glucose levels were significantly lower on day 56 after transplantation of IPCs (12.5 ± 4.7 mmol/L vs 42.2 ± 9.3 mmol/L, P < 0.05).

CONCLUSION: Our results show that UCMSCs can differentiate into islet-like cells in vitro under certain conditions, which can function as IPCs both in vivo and in vitro.
INTRODUCTION
Pancreatic adenocarcinoma is a highly malignant and invasive disease. It has the worst prognosis of all gastrointestinal malignancies[1]. Pancreatic resection plays an essential role in the treatment of pancreatic adenocarcinoma and is the only treatment with the potential to achieve long-term survival[2]. The most common operation for pancreatic neoplasia is pancreaticoduodenectomy. The Achilles heel of this operation is the pancreatic anastomosis, with an incidence of pancreatic leak ranging from 2%-29%[3]. The prognosis remains poor even in patients undergoing radical resection, and there is a median overall survival period of about 20 mo due to the high rate of relapse[4]. Theoretically, elimination of high risk pancreatic anastomosis by performing total pancreatectomy (TP) could reduce perioperative morbidity. Thus, TP has attracted more attention.

TP for pancreatic adenocarcinoma was first reported in 1943 and the patient died in the perioperative period because of a bile duct leak[5]. It was abandoned for a long time because of associated brittle diabetes. In the past decades, TP has shown a definite effect, since remarkable improvements have been achieved in both surgery and postoperative management of endocrine insufficiency of the apancratic patient[6,7]. It is well known that islet transplantation can decrease the morbidity related to diabetes mellitus (DM) after TP. However, the problem of the worldwide lack of donor has yet to be resolved. Moreover, there still exist the seemingly inevitable problems which doctor and patient need to face: immune rejection and recurrent attacks against islets by the underlying autoimmunity[8,9]. Therefore, there is an urgent need for developing alternative cellular therapy strategies for DM.

In the search of new cell sources for transplantation, stem cells represent a critical alternative and would provide a potentially countless source of islet cells for transplantation. Mesenchymal stem cells (MSCs) isolated from human umbilical cord Wharton’s jelly are more primitive MSCs than those isolated from bone marrow or adipose tissue and do not express the major histocompatibility complex (MHC) class II (HLA-DR) antigens[10,11]. Some groups have isolated MSCs from human umbilical cord Wharton’s jelly and induced them to differentiate into islet-like cell clusters[12,13,14].

To solve the islet shortage problem, we tried to induce MSCs isolated from human Wharton’s jelly to differentiate into IPCs in this study. To imitate the pathophysiological status of diabetic patients after TP, we used Sprague-Dawley rats after 90% pancreatectomy as a model of type 1 DM. We for the first time tested the possible curative effects of transplanting IPCs differentiated from umbilical cord mesenchymal stem cells into the model rats.

MATERIALS AND METHODS

Statement of ethics
This study involving the use of SD rats was conducted in strict accordance with provisions and general recommendation of Chinese Experimental Animals Administration Legislation (Permit number SCXK-2010-0001). The cords were collected from obstetrical department of Hua’ian First People’s Hospital with the informed consent of the tissue donor, and following the ethical and institutional guidelines.

Isolation of UCMSCs
With consent from the parents, fresh cords were obtained after birth and collected in normal saline (NS) at 4 ℃. Following disinfection in 75% ethanol for 30 s, the umbilical cord vessels were torn off in NS. After the removal of the supematant fraction, the mesenchymal tissue was diced into cubes and then crushed using serrated thumb forcesps. The mince structure was washed with DMEM (Gibco, Carlsbad, CA, United States) and centrifuged at 200 × g for 5 min. The mesenchymal tissue was digested with collagenase I (Gibco) at 37 ℃ for 1 h and further treated with 0.25% trypsin (Gibco) at 37 ℃ for 30 min. To neutralize the excess trypsin, fetal bovine serum (FBS, Gibco) was added to the mesenchymal tissue. The cells from the two enzymatic digestion steps were combined and counted using a hemocytometer. The mesenchymal cells were then used directly for cultures, and the medium was changed every third day.

Flow cytometry analysis
After the third passages, UCMSCs were released by...
trypsinization. Mouse anti-human antigens CD13, CD34, CD45, CD90, CD105 and HLA-DR were acquired from BD Sciences (Shanghai, CHINA). A total of 1 × 10^6 cells were incubated with PE- or FITC-conjugated antibodies for 20 min at room temperature. Mouse IgG-PE and mouse IgG-FITC were used as isotype controls. The fluorescence intensity of the cells was analyzed using a flow cytometer (FACScan; BD Sciences), and the data were further analyzed using CELLQUEST Pro software (BD Sciences).

Pancreatic endocrine differentiation
For pancreatic differentiation, UCMSCs from fourth passage reaching 80%-90% confluence were induced to differentiate into IPCs. The pancreatic inductive procedure for UCMSCs was performed according to a previous study[6]. Cells were cultured in DMEM/F12 (Gibco) medium containing 10% FBS, 10 mmol/L nicotinamide (Sigma-Aldrich, St.Louis, MO, United States), 4 nmol/L activin-A (Sigma-Aldrich) and 25 ng/mL epidermal growth factor (EGF, PeproTech, Rocky Hill, NJ, United States) for 1 wk. Then the culture medium was changed to DMEM/F12 for another week. Finally, 10 mmol/L nicotinamide, 10 ng/mL of basic fibroblastic growth factor (bFGF, PeproTech) and insulin/transferrin/selenium (ITS, Gibco) were added, and incubation was continued for 2 wk.

DTZ staining
DTZ (Sigma-Aldrich) solution was prepared as reported previously by dissolving 10 mg of DTZ in 1 mL dimethylsulfoxide (DMSO, Sigma-Aldrich) and reserved at -20 °C. The stock DTZ solution was filtered through a nylon filter before use, and for staining 10 μL of the solution was added to 1 mL of cell culture medium. The culture dishes were incubated for 30 min at 37 °C in DTZ-containing solution and then were washed three times with NS. An inverted light microscope was used to examine the color of the clusters and the dishes were then incubated with DMEM containing 10% FBS.

C-peptide secretion measurement
After 2, 3 and 4 wk of induction, cells were rinsed twice with NS and incubated in L-DMEM (5.5 mmol/L glucose, Gibco) for 2 h. C-peptide levels in the culture medium were measured using a C-peptide ELISA kit (Cusabio, Barksdale, DE, United States). TMB substrate was used with absorbance read at 450 nm.

Glucose stimulating test
After 4 wk of cultivation, the differentiated cells were washed twice with NS and incubated in L-DMEM for 2 h. The culture medium was collected and the cells were then washed twice with NS, incubated for 2 h in H-DMEM (25 mmol/L glucose, Gibco), and the culture medium was collected again. C-peptide levels were measured as above.

RT-PCR and real-time RT-PCR analysis
Total RNA was extracted from the cells using TRizol reagent (Invitrogen, Grand Island, NY, United States) according to the manufacturer’s instructions. Gene expression levels of insulin, PDX1, Pax4, Glut2 and Ngn3 were determined by RT-PCR or real-time RT-PCR. The cDNA templates were obtained using oligo(dT) primers (Invitrogen) and PrimeScript RTase reverse transcriptase (Invitrogen). The specific primer pairs and conditions are showed in Table 1.

Experimental animals and 90% pancreatectomy
Male SD rats at 6 wk age weighing 150-160 g were used. The rats were raised on a 12-h dark and 12-h light cycle (darks at 19:00) and fed ad libitum. Rats underwent surgery to either resect 90% of the pancreas (Px) or to have a sham procedure, as reported previously[14]. After the rats of the Px group were anesthetized with 10% chloral hydrate, a middle abdominal incision was made from the xyphoid to hip level. The gastric, splenic and duodenal regions of the pancreas were removed by using cotton tip applicators, leaving all major vessels intact to not compromise the surrounding organs. The area extending from the duct

Table 1 Sequences of reverse transcription-polymerase chain reaction and real-time reverse transcription-polymerase chain reaction primers

| Gene     | Sequence                        | Fragment length (bp) | Annealing temperature (℃) |
|----------|---------------------------------|----------------------|---------------------------|
| Insulin  | F: 5’- ATC AAG CAG ATC ACT GTC CTT CT-3’ R: 5’- GAG AGC TTC CAC CAG GTG TG-3’ | 162                   | 60                        |
| PDX1     | F: 5’- TCC CAT GGA TGA AGT CTA CC-3’ R: 5’- TGT CCT CCT CTT TTC GCC AC-3’ | 246                   | 60                        |
| Ngn3     | F: 5’- CGG CGG TAG AAA GGA TGA C-3’ R: 5’- GAG TGG AGG TGG TGC ATT CC-3’ | 316                   | 60                        |
| Glut2    | F: 5’- AGT ACA ATG ACA GAA CAT AAG GTC-3’ R: 5’- AGC TCC AAC TAA TGA CAG AAT G-3’ | 423                   | 60                        |
| Pax4     | F: 5’- ATC CTT AAG GTA TCT AAT GGC TG-3’ R: 5’- GCC ACT GAA TCA GGA TAC TGC-3’ | 461                   | 60                        |
| GAPDH    | F: 5’- AGA AGG CTG GGG CTC ATT TG-3’ R: 5’- AGG GCC ATC CAC AGT CTT C-3’ | 258                   | 52                        |

F: Forward; R: Reverse.
Cells displayed spindle-shaped, fibroblast-like morphology (A) and they clonally grew and spread at the bottom of a culture dish within the 12\textsuperscript{th} day (B). Flow cytometric analysis showed that the isolated cells at passage 4 strongly expressed the surface markers of mesenchymal stem cells, such as CD90, CD105 and CD13, but almost no markers of hematopoietic stem cells, such as CD34, CD45 and HLA-DR (C). PE: Phycoerythrin; FITC: Fluorescein isothiocyanate.

Figure 1  Isolation and identification of umbilical cord mesenchymal stem cells. Cells displayed spindle-shaped, fibroblast-like morphology (A) and they clonally grew and spread at the bottom of a culture dish within the 12\textsuperscript{th} day (B); Flow cytometric analysis showed that the isolated cells at passage 4 strongly expressed the surface markers of mesenchymal stem cells, such as CD90, CD105 and CD13, but almost no markers of hematopoietic stem cells, such as CD34, CD45 and HLA-DR (C). PE: Phycoerythrin; FITC: Fluorescein isothiocyanate.

to the first part of the duodenum was classified as the residual pancreas (about 10% of the original pancreatic mass). Animals undergoing surgical spleen removal were put in a sham group. Fasting blood glucose concentrations were measured through the angular vein 4 times (at 08:00) in the first week. Thereafter, measurements were taken twice a week. Body weight was also measured twice a week at 08:00.

Transplantation and physiological monitoring
After two days of surgery, the rats were restrained and 1 × 10\textsuperscript{2} IPCs were injected through the portal vein. The sham control group underwent the injection of only NS. Blood glucose and body weight were measured weekly. On day 56, intraperitoneal glucose tolerance was tested with 10% glucose (2 g of glucose/kg of body weight) administered intraperitoneally.

Statistical analysis
Data from the experiment are expressed as the mean ± standard deviation (SD). Group comparisons were made by Student’s t-test. All statistical analyses were conducted using SPSS 17.0 software program. \( P < 0.05 \) was adopted to show statistical significance.

RESULTS

UC-MSC culture and expansion
The isolated umbilical cord cells showed heterogeneity during the first 5 d of cultivation. The cells were elongated, adherent and spindle-shaped, and the individual colonies displayed fibroblast-like morphology after 48 h of plating (Figure 1A). Many cells then clonally grew and spread at the bottom of culture dish within the 12\textsuperscript{th} day (Figure 1B). Flow cytometric analysis showed that the isolated cells at passage 4 strongly expressed the surface markers of MSCs, such as CD90, CD105 and CD13, but almost no markers of hematopoietic stem cells, such as CD34, CD45 and HLA-DR (Figure 1C).

Morphological changes of UCMSCs after 3-stage differentiation protocol
UCMSCs at passage 5 were induced to differentiate into islet-like cells (Figure 2A) by a 3-stage, 4-wk protocol. Upon exposure to serum-free media for an initial 7 d of induction, the adherent spindle-like cells acquired a round shape and assembled together (Figure 2B, stage 1). During stage 2, the round cells became aggregates and some new islet-like clusters began to appear (Figure 2C). The culture of clusters continued to mature and further differentiated into \( \beta \)-like cells (Figure 2D). At the end of stage 3, differentiation of UCMSCs into IPCs was evaluated through DTZ staining. DTZ specifically binds to zinc ion in insulin molecules, which allowed for the identification of clusters with IPCs, whereas undifferentiated cells were negative (Figure 2E).
C-peptide levels of differentiated cells and stimulation by high glucose

As a marker of residual insulin production, C-peptide was used to represent insulin production by our differentiated cells\(^ {15}\). The levels of C-peptide in the culture media of differentiated cells on weeks 2, 3 and 4 showed that these cells produced more C-peptide than the pre-treatment cells (Figure 2F). To examine whether these IPCs could respond to glucose stimulation, C-peptide level in 4-wk differentiated cells was measured. The result showed that differentiated cells secreted C-peptide in a low glucose concentration (5.5 mmol/L) medium and secreted approximately twice as much in a high glucose concentration (25 mmol/L) medium (Figure 2G). Thus, these differentiated MSCs could respond to changes in glucose concentration by altering their C-peptide production and, presumably, insulin production.

Genetic analysis of the differentiated cells

To determine whether the UCMSCs had been induced to differentiate into pancreatic endocrine cells, gene expression profiles for pancreatic \(\beta\)-cell differentiation markers and hormones were assayed by RT-PCR.
and real-time RT-PCR. As illustrated in Figure 3, the inductive cells expressed genes of pancreatic β-cell markers PDX1, Pax4, Glut2, Ngn3 and insulin. However, the expression of PDX1, Ngn3, and insulin in differentiated IPCs was significantly greater than that in undifferentiated MSCs (Figure 3).

**Blood glucose and body weight of rats after surgery**

The blood glucose concentrations and body weight of Px rats and sham rats over the 8 wk of study are shown in Figure 4A and B. The blood glucose levels of Px rats elevated two days after surgery ($P < 0.05$) and the values kept elevating throughout the experimental period (Figure 4A). Body weight of Px rats was significantly lower than that of sham rats by day 17 of the protocol and remained significantly less than sham rats throughout the rest of the experimental period (Figure 4B).

**Effects of cell transplantation on blood glucose and body weight of SD rats**

We sought to determine if the differentiated IPCs had any effects when transplanted into SD rats. In the cell transplantation group, blood sugar levels were significantly lower than in the sham group 2 wk after transplantation (Figure 4C) and body weights were significantly higher at 3 wk after transplantation (Figure 4D). The insulin levels in Px rats transplanted with IPCs for two weeks were increased and maintained to week 8 after transplantation (Figure 4E).

**Transplantation of IPCs improves glucose tolerance in rats after 90% pancreatectomy**

To further estimate the function of the transplanted MSCs, glucose tolerance tests were done on day 56 after transplantation. After intraperitoneal glucose injection, blood glucose values in rats that had been transplanted with IPCs were significantly lower compared to rats that received NS (Figure 4F). In contrast, blood glucose levels remained elevated in rats that received NS. Collectively, these results indicated that transplantation of IPCs led to superior glucose control and tolerance as compared to controls.

**DISCUSSION**

Stem cell regeneration is a promising therapy for patients with insulin dependent DM after TP. Since multipotent mesenchymal stromal cells are free of ethical and immunological complications, they could provide unprecedented opportunity as starting material to derive insulin secreting cells. Many studies have demonstrated the low immunogenicity and pluripotency of UCMSCs\(^{[10,11]}\). Thus, these cells are expected to be an ideal source for transplantation. The phenotypic characteristic of the UCMSCs isolated in our study was in accordance with previous studies\(^{[16]}\). Indeed, the UCMSCs were positive for surface antigens CD13, CD90, and CD105 and negative for surface antigens CD34, CD45 and HLA-DR.

Our results illustrate that UCMSCs could be differentiated into IPCs under specific conditions. The pancreatic inductive protocol for UCMSCs was performed according to a previous study\(^{[6]}\). The adherent spindle-like cells acquired a round shape and assembled together after 1 wk of induction. Some new islet-like clusters began to appear after 2 wk of induction. DTZ staining is a method to identify IPCs, as reported previously\(^{[17,18]}\). DTZ is a substance that can bind to zinc. Compared with other cells, pancreatic islets have higher zinc contents. They become crimson red after DTZ staining\(^{[19]}\). As control cells were negative for...
DTZ staining, our results demonstrated that insulin has been produced by differentiated cells. We used human C-peptide to represent insulin secretion by differentiated cells due to the controversy surrounding insulin uptake by cells from medium supplements [20, 21]. As the precursor of insulin, proinsulin is comprised of three segments: A-chain, B-chain, and C-peptide. However, unlike the A- and B-chains, cells cannot take up C-peptide, so C-peptide levels can be regarded as a marker of insulin production. In our studies, differentiated cells secreted more C-peptide than control cells after 2, 3 and 4 wk of induction. The islet-like clusters were found to be responsive to glucose challenge as evidenced by an around 1.5-fold increase in C-peptide secretion over basal stimulation. It indicated the ability of islet generated to synthesize, store and release insulin in response to glucose challenge. In our in vitro studies, RT-PCR and real time RT-PCR were used to examine the expression of β-cell development-related genes before and after induction of differentiation. After 3 wk of differentiation, the IPCs expressed the genes of pancreatic β-cell markers PDX1, Pax4, Glut2, Ngn3 and insulin. Real-time RT-PCR showed that the expression of PDX1, Ngn3, and insulin was significantly greater in differentiated IPCs than in undifferentiated MSCs.

According to a previous study [14], we created the experimental diabetic rat models by removing 90% of the pancreas. We then transplanted the differentiated cells into diabetic rats in order to examine the function of IPCs. The portal vein [22], renal

Figure 4 Blood glucose and body weight of rats after surgery. Fed blood glucose levels and body weight in Px and sham rats following surgery A: Blood glucose level was higher in Px vs sham on day 2 following surgery and throughout the protocol (P < 0.05); B: Body weight was lower in Px vs sham on day 17 following surgery and throughout the rest of the protocol (P < 0.05); Px (n = 10); sham (n = 8); C: Blood glucose level changes of sham-treated controls (n = 8) and rats that received cell transplantation (n = 6); D: Body weight changes; E: Serum insulin levels. Results are mean ± SE. *P < 0.05 vs controls; F: Improvements in glucose tolerance in diabetic Sprague-Dawley rats transplanted with mesenchymal stem cells. At the indicated time points, blood glucose levels were measured in samples from the angular vein using a glucometer. Results are mean ± SD (n = 6).
In the search of new cell sources for transplantation, stem cells represent a critical alternative and would provide a potentially countless source of islet cells for transplantation. Some groups have isolated MSCs from human umbilical cord Wharton’s jelly and induced them to differentiate into islet-like cell clusters. To solve the islet shortage problem, we tried to induce MSCs isolated from human Wharton’s jelly to IPCs in this study. To imitate the pathophysiological status of diabetic patients after TP, we used SD rats after 90% pancreatectomy as a model of type 1 DM.

**Innovations and breakthroughs**

The authors for the first time examined the possible curative effects of transplanting IPCs differentiated from UCMSCs in SD rats after 90% pancreatectomy.

**Applications**

Based on the effect of reducing blood sugar levels of IPCs differentiated from UCMSCs, this study suggests the therapeutic potential of insulin producing cells in diabetic mellitus.

**Terminology**

UCMSCs are a kind of stem cells isolated from human Wharton’s jelly. TP is an operation containing resection of all pancreatic tissue.

**Peer-review**

The manuscript discusses a very interesting topic. In addition, the manuscript is well written. It presents the results in a clear and well explained manner both in text and in figures.

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