

Transplantation of Insulin-Producing Clusters Derived From Adult Bone Marrow Stem Cells to Treat Diabetes in Rats

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Abstract

Objectives: Recent findings suggest that bone marrow stem cells can differentiate into numerous cell types. This would provide a potentially unlimited source of isletlike cells for transplantation and a promising therapy for diabetes mellitus. Here, we studied the differentiation ability of adult bone marrow hematopoietic-rich stem cells to form glucose-regulating insulin-producing cells. Their ability to treat chemically induced diabetes in rats was then tested.

Materials and Methods: Hematopoietic-rich stem cells were obtained from the long bones of rats and cultured in a serum-free medium containing 1% dimethyl sulfoxide for 3 days. The cells were cultured for 7 days in a glucose-rich medium supplemented with pancreatic extract. Thereafter, cultures were done in a medium (low concentration of glucose and 5% fetal bovine serum) supplemented with nicotinamide and exendin-4 for 7 more days.

Results: At day 17 of culture, the cells formed isletlike clusters. These were distinctly stained crimson red by diphenylthiocarbazone and expressed insulin and endocrine-specific transcription genes. Insulin was secreted in a dose-response manner as a function of increasing glucose concentrations. When transplanted in the testes of diabetic rats, the differentiated cells could

normalize blood glucose levels for 3 months in 80% of the treated rats. The therapeutic benefits were reversed after orchidectomy.

Conclusions: Hematopoietic-rich stem cells may include pancreatic progenitor cells capable of differentiating into functioning endocrine hormone-producing cells. This finding suggests a possible means of treating diabetes mellitus.

Key words: Diabetes mellitus, Cell therapy, Insulin-secreting cells

Diabetes mellitus is a devastating disease affecting millions of people worldwide. Maintaining good glycemic control with exogenous insulin imposes an enormous burden on patients. An alternative treatment for patients with type 1 diabetes is whole organ pancreatic transplant. Such a procedure offers the possibility of excellent glycemic control; however, patients are subjected to the adverse effects of immunosuppression and the risks of major surgery. Accordingly, transplanting the pancreas is done only when patients receive a solid organ transplant. Islet transplanting also offers the possibility of internal glycemic control and does not subject patients to a major surgical procedure (1). However, islets derived from multiple donors are required to achieve insulin independence. Immunosuppression is necessary, and the current shortage of deceased-donor organs had limited the wide use of this approach.

Recent studies have shown that adult bone-marrow-derived stem cells can differentiate into several cell types such as blood, liver, lung, skin, muscle, neuron, and insulin-producing cells (2-7). This has led many investigators to explore the potentials of their therapeutic applications.

The aim of this research was to isolate adult bone marrow hematopoietic-rich stem cells and induce

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them to differentiate into insulin-producing cells in vitro. After characterization, the differentiated cells were tested for their ability to treat chemically induced diabetic rats.

Materials and Methods

Experimental animals and study groups

Forty adult (3-month-old) inbred Sprague-Dawley male rats weighing an average of 150 g were divided into 4 experimental groups of 10 animals each. Animals in "group nondiabetic control" underwent a sham experiment. Diabetes was chemically induced in the remaining animals using an intravenous injection of streptozotocin (50 mg/kg body weight). Blood glucose levels were monitored with an Accutrend glucose detector (Boehringer Mannheim GmbH, Mannheim, Germany). Rats with 2 successive blood glucose levels higher than 350 mg/dL were further divided as follows: "group acellular" received acellular tissue culture medium; "group undifferentiated" received undifferentiated stem cells, and "group differentiated" was treated with the differentiated cells. All protocols were approved by the institution's animal welfare regulatory committee.

Isolation and in vitro differentiation of hematopoietic-rich cells

Isolation and in vitro differentiation of bone marrow stem cells were done as described previously (6) with some modifications. Bone marrow was obtained from the long bones of adult Sprague-Dawley rats. For each experiment, bone marrow cells were isolated from 4 donor rats. The bones were sterilized by immersion in 70% ethanol. The ends of the bones were cut, and bone marrow was extruded by inserting a needle in 1 end through the bone shaft and injecting tissue culture medium (Dulbecco's modified Eagle's medium; Sigma Chemical Company, St. Louis, MO, USA) containing 10% fetal bovine serum (Sigma). The effluent was collected in sterile tubes. Gentle pipetting resulted in generation of a single-cell suspension. Bone marrow cells were counted and plated with a concentration of 10×10^6 /mL in T-75 flasks. Cells were then cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μ g/mL). After 60 minutes of incubation, nonadherent cells were collected and

washed with fresh serum-free medium. These cells were re-plated in plastic 6-well plates on slide cover slips (22×22 mm²) coated with 0.3% type 1 collagen at a cell density of 20×10^6 /well in serum-free medium (3 mL/well) containing 1% dimethyl sulfoxide and cultured for 3 days. The medium was then replaced by one containing 25 mM glucose, 10% fetal bovine serum, and 200 μ g/mL pancreatic extract for 7 days. To enhance the sensitivity of the differentiated clusters to glucose challenge, the culture medium was changed to contain 5.5 mM glucose, 5% fetal bovine serum, 10 mM nicotinamide (Sigma), and 10 nM exendin-4 (Sigma), and cultured for 7 more days.

Preparation of pancreatic extract

Pancreatic extract was prepared according to the method described by Choi and associates (8). Five-week-old Sprague-Dawley rats were anesthetized using ketamine HCl (60 mg/kg) and pentobarbital-Na (20 mg/kg). The splenic portion of the pancreas was removed. After 48 hours, rats were killed by cervical dislocation and immediately dissected to remove the regenerating pancreas. The excised tissues were placed in chilled phosphate buffered saline containing protease inhibitor complex (1 mL/1 mg tissue) and homogenized. Homogenates were centrifuged at 1700 g for 10 minutes at 4°C and then at 28 000 g for 20 minutes also at 4°C. The final clear supernatant was analyzed for the protein content using the Bradford method. The extract was then stored as aliquots at -70°C until further use.

Viability testing and functional evaluation of the differentiated clusters

At day 17 of culture, the differentiated clusters were examined for viability by trypan blue exclusion.

The ability of the clusters to produce insulin was tested by staining with diphenylthiocarbazone (DTZ, zinc-chelating agent). Stock solution was prepared as previously described (9). Diphenylthiocarbazone 50 mg (Sigma) was dissolved in 5 mL dimethyl sulfoxide and stored at -20°C. For staining, 10 μ L of the stock solution was added to 1 mL of the culture medium. Cells were incubated at 37°C for 30 minutes in the prepared medium. Cells were then examined with a stereomicroscope after rinsing the plates 3 times with Hank's balanced salt solution (Sigma).

Cytospin slides from undifferentiated and differentiated hematopoietic-rich stem cells were

fixed with 4% paraformaldehyde in phosphate buffered saline. After washing 3 times with phosphate buffered saline, the slides were treated with monoclonal rat anti-insulin antibody (Zymed Laboratories, Invitrogen immunodetection, San Francisco, CA, USA) or polyclonal rabbit anti-rat C-peptide antibody (Cell Signaling Technology, Inc, Danvers, MA, USA). Immunoreactive cells were visualized using a Vectastain Elite ABC Kit (Vector Laboratories, Inc., Burlingame, CA, USA) with 3:3 diaminobenzidine tetrachloride as the chromogen. Cells were then counterstained with hematoxylin & eosin stains.

Determination of insulin secretion

Undifferentiated and differentiated bone marrow cells were initially incubated for 3 hours in glucose-free Krebs-Ringer bicarbonate buffer (KRB) containing 0.5% bovine serum albumin. This was followed by incubation in KRB containing 5.5, 12, or 25 mM glucose concentration for an additional 2 hours. The KRB was collected and frozen at -70°C until assayed. Insulin assay was done by enzyme-immunoassay (Linco Research Inc, St. Charles, MO, USA) according to the manufacturer's instruction.

Determining the intracellular C-peptide content

The formed clusters were washed 3 times with phosphate buffered saline. They were then suspended and dispersed in 50 mM HCl/70% ethanol. After centrifugation at 12000 g for 5 minutes, the supernatant was collected from the cell lysate and neutralized by adding 50 mM NaOH. C-peptide concentrations in the supernatants were determined by a rat C-peptide ELISA kit (Gentaur molecular products BVBA, Legerlaan, Brussels) according to the manufacturer's instructions.

Detection of islet-related gene expression

Gene expression levels of insulin1, glucagon, somatostatin, pancreatic polypeptide, pancreatic-duodenal homeobox1 (PDX-1), NeuroD1, glucose transporter gene (Glut-2), and paired box gene (PAX-6) were determined by reverse transcription polymerase chain reaction (RT-PCR). Glyceraldehyde 3-phosphate dehydrogenase was included as an internal control. Primer sequences for RT-PCR are shown in Table 1. Total RNA was extracted from adult rat pancreas, as well as from undifferentiated and differentiated bone marrow cells with TRIzol reagent according to the manufacturer's instructions (Invitrogen Corporation, Grand Island, NY, USA). Reverse transcription was done using 1 μg total RNA and a cDNA kit (high-capacity cDNA archive kit: ABI PRISM 3100 Genetic Analyzer [Applied Biosystems, Foster City, California, USA]). Two μL of the cDNA sample was mixed with 1 μL of each primer and 50 μL of Taq PCR (master mix kit, QIAGEN Inc, Valencia, CA, USA). Distilled water was added to a volume of 100 μL , and the resulting mixture was subjected to PCR amplification. The cycling parameters were as follows: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds, annealing at 53°C to 57°C (depending on the primer) for 30 seconds, elongation at 72°C for 30 seconds, and final extension at 72°C for 10 minutes. The resulting products were electrophoresed in a 1% agarose gel to detect gene bands and photographed with a Kodak digital camera.

Transplantation of differentiated isletlike clusters in rats

Differentiated isletlike clusters were transplanted in abdominally placed testes as 5000 clusters per rat (10 rats). Through a lower abdominal incision, the testes

Table 1. List of rat gene-specific primers in RT-PCR.

Genes	Forward primer	Reverse primer	Anneal temp ($^{\circ}\text{C}$)	Size of PCR product (bp)
Insulin1	CCGTCGTGAAGTGGAG	CAGTTGGTAGAGGGAGCAG	57	156
Glucagon	ATCATTCCCAGCTCCAGA	CGGTTCTCTGGTGTCAT	54	152
Somatostatin	CAGGAAGTGCCCAAGTAC	AGTTCCTGAGCCAGCTTTG	54	187
P. polypeptide	CGCATACTACTGCCTCTCCC	CAGCAGCGCAGGGCATCAA	53	278
PDX-1	GGTGCCAGAGTTCAGTGCTAA	CCAGTCTCGGTTCCATTG	53	249
NeuroD1	TGTCGTTACTGCCTTTGGAA	CGATCTGAATACAGTACACGAA	53	151
GLUT-2	TTACTCTCCATTTCAGTCCTTTGT	TAGAGCAGCTCTTTATTCCAGATT	53	165
PAX-6	CGACAAGATTGGCATGGAT	CAACCTTTGAAAAACCAACA	54	179
GAPDH	CACCCTGTTGCTGTAGCCATATTC	GACATCAAGAAGGTGGTGAAGCAG	57	196

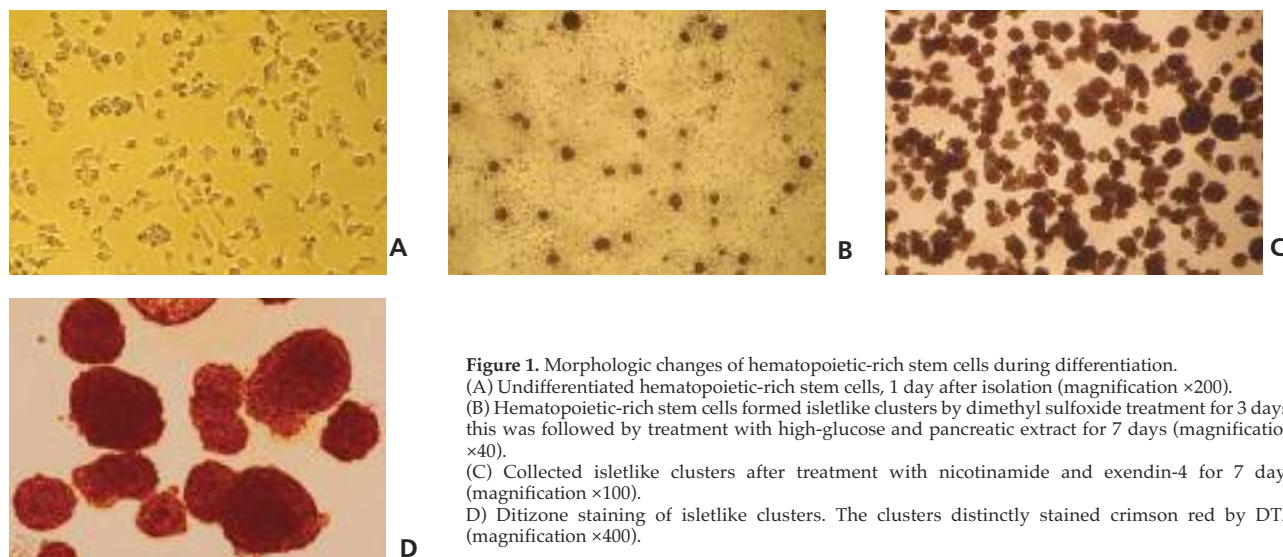


Figure 1. Morphologic changes of hematopoietic-rich stem cells during differentiation. (A) Undifferentiated hematopoietic-rich stem cells, 1 day after isolation (magnification $\times 200$). (B) Hematopoietic-rich stem cells formed isletlike clusters by dimethyl sulfoxide treatment for 3 days; this was followed by treatment with high-glucose and pancreatic extract for 7 days (magnification $\times 40$). (C) Collected isletlike clusters after treatment with nicotinamide and exendin-4 for 7 days (magnification $\times 100$). (D) Ditzone staining of isletlike clusters. The clusters distinctly stained crimson red by DTZ (magnification $\times 400$).

were pulled through the inguinal canal to the abdominal cavity. Clusters were injected into the testis through a 26-gauge needle. The grafted gonad was anchored to the abdominal wall by suturing the gubernaculum to the abdominal muscles.

Follow-up evaluation

Urine output of the experimental animals was measured daily, and their weights were determined every week. Random blood glucose levels and insulin assays were determined 1 week after transplant and every 2 weeks thereafter.

Orchidectomy of the engrafted testes

The gonads of rats that had undergone a transplant were surgically removed 3 months after transplant or after the animal's death. This was followed by further measurements of glucose and insulin levels. The excised gonads were fixed with 10% neutral formalin and processed to obtain paraffin blocks. Sections were stained with the same anti-insulin and anti-rat C-peptide antibodies as previously described.

Postmortem evaluation

At the end of the observation period, rats were killed, and their pancreata harvested and immunohistochemically stained for insulin and C-peptide as previously described.

Statistical analyses

Data are presented as means $\pm$ SD. Statistical differences were measured using the 2-tail Kruskal-Wallis H test. Statistical significance was set at $P < .05$.

Results

Differentiation of hematopoietic-rich stem cells in culture

Hematopoietic-rich stem cells obtained and cultured as described underwent a series of changes as shown in Figures 1A through 1C. At the end, cell aggregates that mimicked an islet architecture became compact and formed isletlike clusters. The mean number of clusters formed on each cover slip was 500 ± 45 .

Viability testing and functional evaluation of the differentiated clusters

The viability of the formed clusters was confirmed by trypan blue exclusion. The isletlike clusters were distinctly stained crimson red by DTZ (Figure 1D). However, the undifferentiated cells did not. Moreover, the clusters were positively stained with both insulin and C-peptide antibodies (Figure 2). In contrast, undifferentiated cells did not stain.

Insulin release by, and C-peptide content in, differentiated isletlike clusters

When exposed to increasing glucose concentrations, the isletlike clusters secreted increasing amounts of insulin in a glucose concentration-dependent manner (Figure 3). The amount of insulin secreted in 25 mM glucose by 100 clusters was nearly 6 times that of the 5.5-mM glucose concentration. When the glucose concentration was 25 mM, the content in the same number of clusters ranged between 150 and 190 ng/mL for insulin and 960 and 1038 pg/mL for C-peptide.

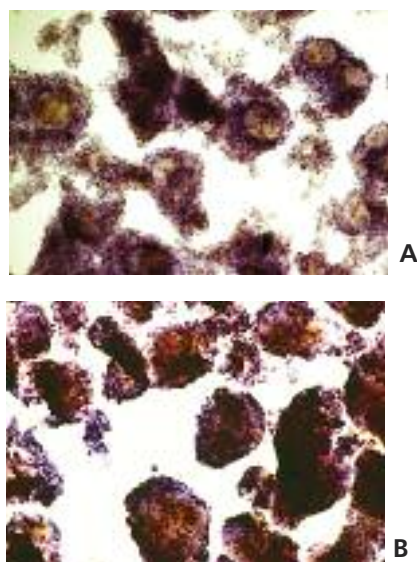


Figure 2. Immunocytochemical staining of hematopoietic-rich stem cells for insulin and C-peptide. (A) Differentiated cells (positive for insulin, magnification $\times 100$). (B) Differentiated cells (positive for C-peptide, magnification $\times 100$).

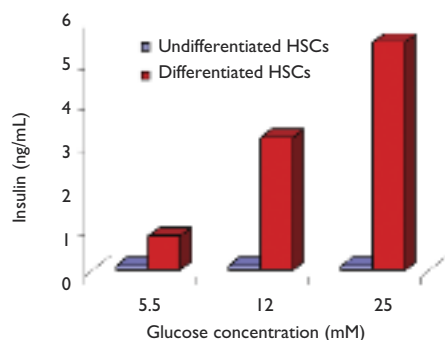


Figure 3. Insulin release in response to glucose stimulation as detected by immunosorbent assay. The mean insulin secretion by 100 clusters was 0.83 ± 0.05 ng/mL in response to 5.5 mM glucose; it was 3.2 ± 0.61 ng/mL in response to 12 mM; and it was 5.5 ± 0.5 ng/mL when the 25-mM glucose concentration was used. These results represent the mean of 6 experiments; differences were statistically significant ($P < .05$).

Gene expression in differentiated isletlike clusters

The reverse transcriptase-polymerase chain reaction products of insulin1, glucagon, somatostatin, and pancreatic polypeptide genes were expressed in the differentiated clusters. Transcripts of PDX-1, NeuroD1, Glut-2, and PAX-6 were also up-regulated in these clusters (the marker used was 100 bp). Gene expression analysis in isletlike clusters was similar to that of pancreatic tissue and was not demonstrated in undifferentiated hematopoietic-rich stem cells (Figure 4).

Transplanting differentiated isletlike clusters in rats

Figure 5 shows the profiles of the 4 experimental groups relative to blood glucose and serum insulin

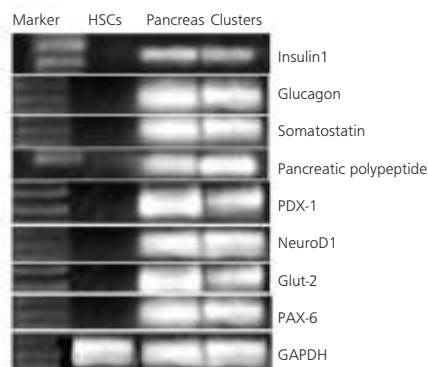


Figure 4. Gene expression of undifferentiated hematopoietic-rich stem cells, rat pancreas (control), and isletlike clusters. The figure is representative of 1 of 4 experiments.

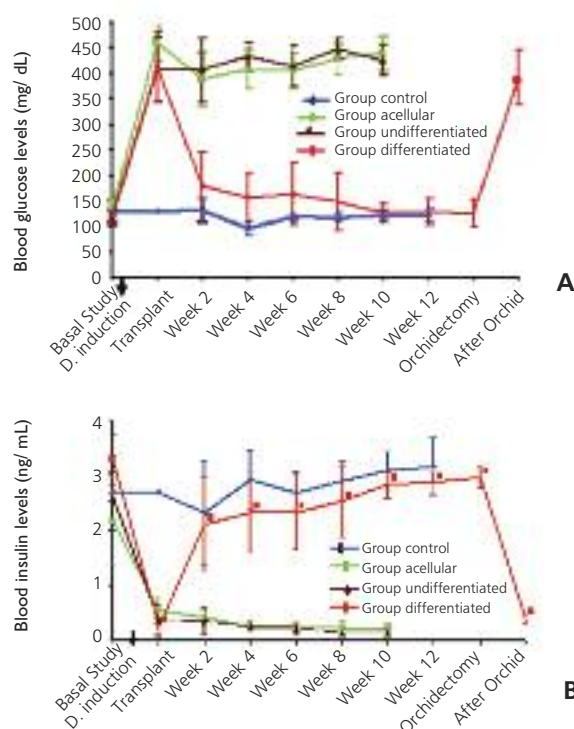


Figure 5. (A) Changes in the glucose concentrations among the 4 studied groups: group control (sham experiment), group acellular (diabetic rats treated by culture media), group undifferentiated (diabetic rats treated by undifferentiated hematopoietic-rich stem cells), and group differentiated (diabetic rats treated by differentiated clusters).

(B) Changes in the insulin levels in clusters of rats that had undergone a transplant.

Blood glucose began to decrease within 2 to 3 days. Throughout the observation period, the determined blood glucose levels were significantly lower ($P < .001$) and serum insulin levels were significantly higher ($P < .001$) in animals in group differentiated than they were in animals in group acellular and group undifferentiated. Following orchidectomy, these rats became hyperglycemic again with a steep reduction in the serum insulin levels.

levels throughout the observation period. Hyperglycemia could be reversed in 8 of 10 diabetic rats that received differentiated insulin-producing cells. The blood glucose began to decrease within 2 to 3 days. Throughout the observation period, the

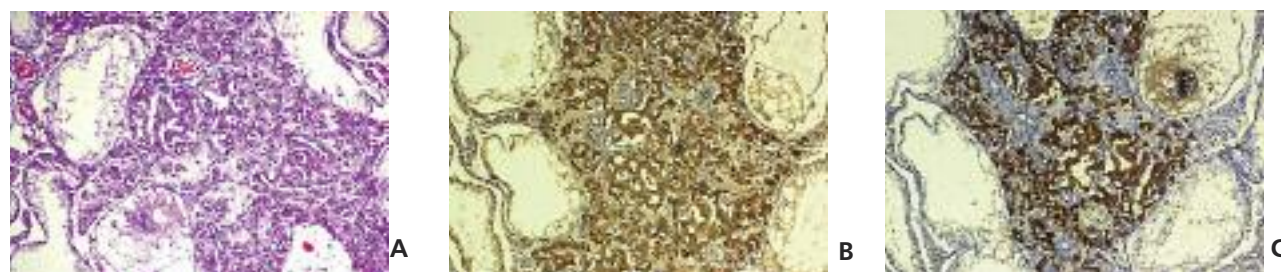


Figure 6. Histology of the grafted testes.

(A) Hematoxylin and eosin staining of the engrafted clusters.

Shows intact cells within the interstitial tissue. No lymphocyte infiltration was observed (magnification $\times 100$).

(B) Immunohistochemistry for insulin expression of the engrafted clusters was positive (magnification $\times 100$).

(C) Immunohistochemistry for C-peptide expression reveals that most cells of the grafted clusters were positive (magnification $\times 100$).

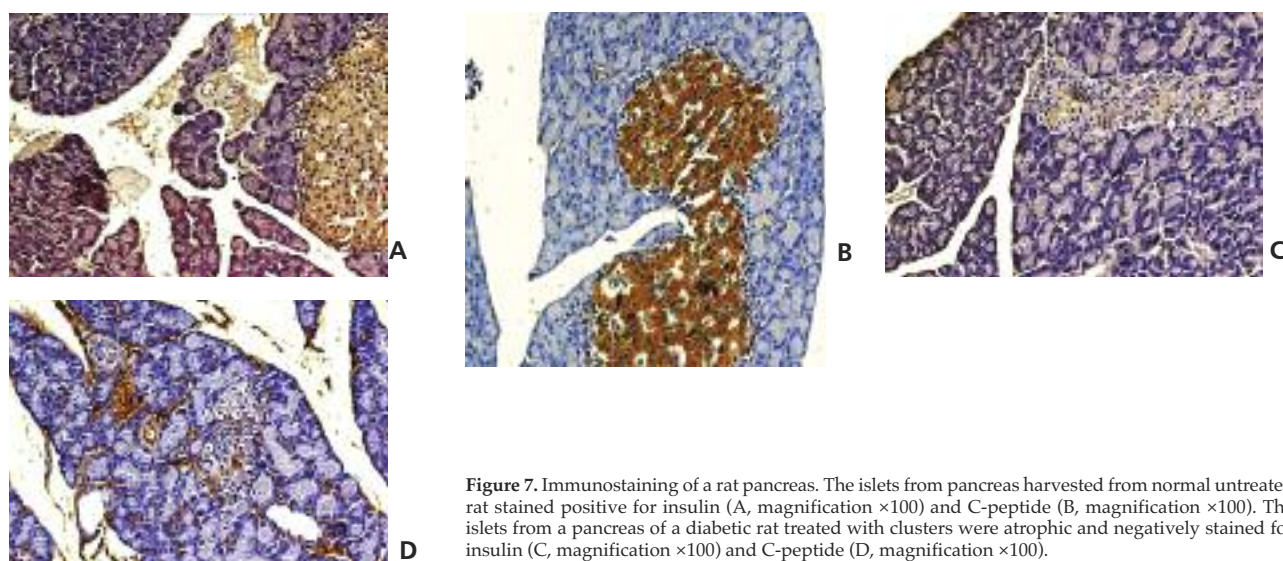


Figure 7. Immunostaining of a rat pancreas. The islets from pancreas harvested from normal untreated rat stained positive for insulin (A, magnification $\times 100$) and C-peptide (B, magnification $\times 100$). The islets from a pancreas of a diabetic rat treated with clusters were atrophic and negatively stained for insulin (C, magnification $\times 100$) and C-peptide (D, magnification $\times 100$).

determined blood glucose levels were significantly lower ($P < .001$), and serum insulin levels were significantly higher ($P < .001$) in animals in group differentiated than they were in animals in group acellular and group undifferentiated. An increase in body weight and a reduction in the urine volume of the treated animals (group differentiated) also was noted. After orchidectomy, these rats became hyperglycemic again with a steep reduction in the serum insulin levels.

Immunohistochemistry studies

Histology of the testes of rats that received the differentiated clusters revealed that the cell aggregates were arranged within the interstitial tissue. There was no lymphocytic infiltrate within the testicular tissue or in the transplanted clusters. In addition, the transplanted clusters were positively stained for insulin and C-peptide by immunohistochemistry (Figure 6).

In contrast to the findings in a normal pancreas, harvested pancreata from the treated animals showed atrophy of the islets and negative staining for insulin and C-peptide (Figure 7).

Discussion

Several in vitro studies have shown that bone-marrow-derived stem cells could be reprogrammed to become functionally insulin-producing cells under certain culture conditions (6, 10). The mammalian pancreas develops from the embryonic foregut of the endodermal layer. Differentiation into insulin-producing endocrine cells is induced by a cascade of gene events controlled by several transcription factors such as PDX-1 and PAX-6 (11). Induction of bone marrow stem cells to differentiate into insulin-producing cells is similar to that process (directional strategy).

Initially, PDX-1 gene expression should be

induced using factors such as dimethyl sulfoxide (6), trichostatin A (12), or β -mercaptoethanol (13). Subsequently, the cells are cultured in glucose-rich medium supplemented with pancreatic extract. Glucose is a growth factor for β -cell replication in vitro and in vivo at 20- to 30-mM concentrations. Glucose has been shown to increase the insulin content in cells derived from embryonic stem cells at a 5-mM concentration (14). In effect, glucose could have a dual role. In the proliferation phase, the high glucose content may support the extra energy needed for cell division. In the differentiation stage, it could modulate specific gene programs linked to glucose sensing and insulin secretion. Kim and associates (15) reported that rat pancreatic extract can provide regeneration factors that induce pancreatic regeneration. This finding also was confirmed by Choi and associates (8). Finally, nicotinamide and exendin-4 were added to the culture medium. Nicotinamide is a poly ADP-ribose synthetase inhibitor and could induce pancreatic progenitor cells into insulin-producing cells (16). Whereas, exendin-4 also could stimulate β -cell replication and neogenesis from ductal progenitor cells and inhibit apoptosis of β cells (17).

Gene expression by isletlike clusters should be similar to that of pancreatic endocrine tissue. Insulin production was confirmed by staining with zinc chelating agent (DTZ). These cells were not only capable of insulin production but also of its release in a dose-dependent fashion according to the glucose concentration. Expression of insulin1, glucagon, somatostatin, and pancreatic polypeptide genes could be demonstrated in the differentiated clusters. Evidence also has been provided that PDX-1, NeuroD1, Glut-2, and PAX-6 were up-regulated. Some investigators suggest that some insulin secreted by the differentiated cells may have been derived from insulin added to the culture medium in certain protocols or insulin present in sera. However, the step-wise increase in insulin release as a function of glucose concentration does not support these contentions. In addition, detection of C-peptide in the isletlike clusters, immunohistologically as well as by chemical assay, confirms that insulin release was the result of endogenous synthesis.

Analysis of cellular expression in vitro can help select promising starting material and can offer evidence of progress toward differentiation. However, the capacity of these cells to do glucose-

responsive insulin-producing functions in vivo, is critical to determine their possible therapeutic usefulness. Some factors that complicate in vivo studies include the choice of animal model, immunologic rejection, detection of cells after transplant, and long-term cellular engraftment with physiologically appropriate insulin production.

Insulin-producing cells were used to treat chemically induced diabetes in nonobese diabetic mice by Oh and associates (6) and Tang and associates (16). In the former study, insulin-producing cells were derived from adult bone marrow hematopoietic stem cells and were injected into the kidneys of the diabetic mice. In the latter study, insulin-producing cells were derived from adult bone marrow mesenchymal stem cells and were transplanted in the kidneys and spleens of the diabetic mice.

In our experiment, insulin-producing cells were obtained from adult bone marrow hematopoietic-rich stem cells and were grafted into the testes of inbred rats. Inbred rats were used to circumvent any immunologically related issues. The rats that received the differentiated insulin-producing cells became euglycemic for 3 months. This improvement in blood glucose was associated with a gain in body weight and a reduction in urine volume. The diabetic rats that received acellular medium or undifferentiated cells showed no sign of recovery or improvement in their diabetic status during the observation period. This indicates that there was no spontaneous recovery or regeneration of the pancreatic islet cells in this experimental setting. This also was confirmed by the negative staining for insulin and C-peptide in the harvested pancreata of the treated animal. The testes, as a site for transplant, was chosen for its accessibility and ease of orchidectomy for immunohistologic documentation and to monitor the biochemical changes after removal of the transplanted isletlike cells.

Orchidectomy of the testes bearing insulin-producing cells was a crucial component of our experimental protocol. After orchidectomy, the previously euglycemic rats became hyperglycemic again with steep reduction in serum insulin levels. Immunohistochemistry of the removed testes revealed viable insulin as well as C-peptide staining cell aggregates. Furthermore, we found no evidence of lymphocytic infiltration that would indicate a lack of immune response.

In conclusion, glucose-responsive insulin-producing cells could be obtained from adult hematopoietic bone marrow stem cells of rats. These cells were successfully used to treat diabetic animals. A group of Chinese investigators (13) had reported their success regarding the differentiation of bone marrow mesenchymal stem cells from a diabetic patient into functional insulin-producing cells in vitro. This suggests that use of a diabetic patient's own bone marrow stem cells as a source of autologous insulin-producing cells for β -cell replacement could be feasible.

Several problems (eg, establishing a reproducible protocol) must be solved before this process becomes a clinical reality. Large numbers of insulin-producing clusters produced in vitro must be obtained for transplant. Several growth or differentiating factors should be tested, and better culture conditions must be investigated. Use of the extracellular matrix may be important. The possible advantages of bioreactors also should be explored. The cell product must mimic some phenotypic traits of the mature β -cell, such as glucose sensing, insulin processing, and secretion in appropriate amounts. Expression of autoantigens must be abrogated to avoid regaining the autoimmune response. Lastly, the conditions of, and the site for, transplant of these cells must be optimized.

References

1. Shapiro AM, Lakey JR, Ryan EA, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med*. 2000;343(4):230-238.
2. Krause DS, Theise ND, Collector MI, et al. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell*. 2001;105(3):369-377.
3. Jiang Y, Jahagirdar BN, Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature*. 2002;418(6893):41-49. Erratum in: *Nature*. 2007;447(7146):879-880.
4. Mezey E, Key S, Vogelsang G, Szalayova I, Lange GD, Crain B. Transplanted bone marrow generates new neurons in human brains. *Proc Natl Acad Sci USA*. 2003;100(3):1364-1369.
5. D'Ippolito G, Diabira S, Howard GA, Menei P, Roos BA, Schiller PC. Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. *J Cell Sci*. 2004;117(Pt 14):2971-2981.
6. Oh SH, Muzzonigro TM, Bae SH, LaPlante JM, Hatch HM, Petersen BE. Adult bone marrow-derived cells trans-differentiating into insulin-producing cells for the treatment of type I diabetes. *Lab Invest*. 2004;84(5):607-617.
7. Moriscot C, de Fraipont F, Richard MJ, et al. Human bone marrow mesenchymal stem cells can express insulin and key transcription factors of the endocrine pancreas developmental pathway upon genetic and/or microenvironmental manipulation in vitro. *Stem Cells*. 2005;23(4):594-603.
8. Choi KS, Shin JS, Lee JJ, Kim YS, Kim SB, Kim CW. In vitro trans-differentiation of rat mesenchymal cells into insulin-producing cells by rat pancreatic extract. *Biochem Biophys Res Commun*. 2005;330(4):1299-1305.
9. Shiroy A, Yoshikawa M, Yokota H, et al. Identification of insulin-producing cells derived from embryonic stem cells by zinc-chelating dithizone. *Stem Cells*. 2002;20(4):284-292.
10. Jahr H, Bretzel RG. Insulin-positive cells in vitro generated from rat bone marrow stromal cells. *Transplant Proc*. 2003;35(6):2140-2141.
11. Chakrabarti SK, Mirmira RG. Transcription factors direct the development and function of pancreatic beta cells. *Trends Endocrinol Metab*. 2003;14(2):78-84.
12. Tayaramma T, Ma B, Rohde M, Mayer H. Chromatin-remodeling factors allow differentiation of bone marrow cells into insulin-producing cells. *Stem Cells*. 2006;24(12):2858-2867.
13. Sun Y, Chen L, Hou XG, et al. Differentiation of bone marrow-derived mesenchymal stem cells from diabetic patients into insulin-producing cells in vitro. *Chin Med J (Engl)*. 2007;120(9):771-776.
14. D'Amour KA, Bang AG, Eliazar S, et al. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nat Biotechnol*. 2006;24(11):1392-1401.
15. Kim YS, Lee JJ, Shin JS, Kim HJ, Kim CW. Enhancement of mouse pancreatic regeneration and HIT-T15 cell proliferation with rat pancreatic extract. *Biochem Biophys Res Commun*. 2003;309(3):528-532.
16. Tang DQ, Cao LZ, Burkhardt BR, et al. In vivo and in vitro characterization of insulin-producing cells obtained from murine bone marrow. *Diabetes*. 2004;53(7):1721-1732.
17. Xu G, Stoffers DA, Habener JF, Bonner-Weir S. Exendin-4 stimulates both beta-cell replication and neogenesis, resulting in increased beta-cell mass and improved glucose tolerance in diabetic rats. *Diabetes*. 1999;48(12):2270-2276.