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Original Communication

Biochemical induction of adult bone marrow hematopoietic stem cells into insulin producing cells

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ABSTRACT

The strategy to differentiate stem cells into insulin producing cells (IPCs) *in vitro* is a promising one to get a cell source for β-cell replacement therapy of diabetes. Bone marrow stem cells (BMSc) have the capacity to differentiate into various cell lineages including endocrine cells of pancreas. Herein, full characterization of insulin producing cells derived from adult bone marrow hematopoietic stem cells (HSCs) was studied. HSCs were obtained from the long bones of Sprague Dawley (SD) rats and differentiated into insulin producing cells by using DMSO and pancreatic extract. The differentiated HSCs were distinctly stained crimson red by DTZ and expressed insulin, glucagon, somatostatin and endocrine-specific transcription genes. Immunocytochemistry and Immunofluorescence revealed that the differentiated HSCs were positively stained for insulin and c-peptide. These results were confirmed by flow cytometric analysis, that they were positively stained for insulin. Enzyme-linked immunosorbent assay analysis demonstrated that insulin was secreted in a dose-response fashion as a function of increasing glucose concentrations. These findings suggest that HSCs can differentiate into insulin producing cells similar to the islets of langerhans and could be used for the treatment of diabetes.

KEYWORDS: immunochemistry, diabetes mellitus, hematopoietic stem cells, insulin-secreting cells

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INTRODUCTION

Diabetes Mellitus is defined as a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both. The chronic hyperglycemia is associated with long-term damage, dysfunction and failure of various organs, especially the eyes, kidney, nerve, heart, and blood vessels [1].

Cell therapy, principally islet transplantation, is one important area of research to improve therapy for diabetes. Although some progress has been reported in islet transplantation, it has been hampered by immune rejection as well as the deficiency of transplantable donor islets [2, 3].

One theoretical alternative for islet transplantation is the use of renewable insulin producing cells. Stem cells are biological cells found in all multicellular organisms that can divide and differentiate into diverse specialized cell types and can self-renew to produce more stem cells. In mammals, there are two broad types of stem cells: embryonic stem cells, which are isolated from the inner cell mass of blastocysts, and adult stem cells, which are found in various tissues. In adult organisms, stem cells and progenitor cells act as a repair system for the body, replenishing adult tissues. In a developing embryo, stem cells can differentiate into all the specialized cells (these are called pluripotent cells), but also maintain the normal turnover of regenerative organs, such as blood, skin, or intestinal tissues.

Recent studies showed embryonic stem cells, pancreatic ductal cells, hepatic stem cells and bone

marrow-derived cells as alternative sources of stem cells, capable of differentiation into functional sources of insulin- producing cells [4-7].

Here we focus on adult bone marrow-derived cells because these cells have the plasticity, as well as there is no need to consider ethical problems. Recent studies have shown that adult bone marrow-derived stem cells have the ability to differentiate into a number of cell types such as blood, liver, lung, skin, muscle, neuron and insulin producing cells [8-13].

The aim of this study is to isolate adult bone marrow hematopoietic stem cells and induce them biochemically to differentiate into insulin-producing cells *in vitro*. Subsequently, full characterization and functional evaluation of the differentiated cells were carried out.

MATERIAL AND METHODS

Isolation of hematopoietic cells

Bone marrow was obtained from long bones (femurs and tibias) of Sprague Dawley rats as previously described [14]. The bones were sterilized by immersion in 70% ethanol, followed by removal of the remaining skin and muscles. Bone marrow was exposed by cutting the ends of the bones and extruded by inserting a needle and forcing cell culture medium with 10% fetal bovine serum through the bone shaft. Gentle pipetting resulted in generation of a single cell suspension. Bone marrow cells were re-suspended, counted and plated at the concentration of $10x10^6$ ml in T-75 flask. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Compo St. Lous, USA.) containing 10% fetal bovine serum (FBS, Sigma) and 1% penicillin and streptomycin. After 12 hrs of incubation, non-adherent cells were collected and washed with fresh, serum free medium.

Characterization of isolated HSCs by flow cytometric analysis

For flow cytometric analysis, the methods were done as we previously described [15]. Briefly, the bone marrow-derived HSCs were centrifuged at 300 g for 8 minutes, and then suspended in phosphate-buffered saline PH 7.4 (PBS) at a concentration of 1×10^6 cells/ml. The antibodies against CD34, CD29 and CD106 directly conjugated to phycoerythrin (PE) and CD44 conjugated with fluorescein-isothiocyanate (FITC) (Becton-Dickinson, USA) were added (10 µL for each 100 µL of sample) and incubated for 30 minutes at room temperature. Labeled cells were thoroughly washed with two volumes of phosphate buffered saline (PBS) and fixed in flow buffer (1% formaldehyde in PBS). The labeled cells were then analyzed using an argon ion laser with a wave length of 488 nm (FACS Calibur, Becton-Dickinson, USA). A total of 10000 events were obtained and analyzed with the Cell Quest software program (Becton-Dickinson, USA).

Induction of HSCs into insulin producing cells

Differentiation of HSCs was carried out as we previously described [14]. The isolated non adherent cells were re-plated in plastic six well plates on slide cover slips coated with 0.3% type 1 collagen at a cell density of 20 x 10^6/well in serum free medium containing 1% dimethyl sulfoxide (DMSO) and cultured for 3 days. The medium were then replaced with another medium containing 25 mM/l glucose, 10% fetal bovine serum and 200 ug/ml prepared pancreatic extract then incubated for 7 days. To complete the maturation of the formed clusters, the medium was changed to another medium containing 5.5 mM/l glucose, 5% FBS, 10 mM/l nicotinamide and 10 nM/l exendin 4 and cultured for 7 days.

Preparation of pancreatic extract

Pancreatic extract was prepared according to the method described before [16]. Five-week-old Sprague Dawley rats were partially (60%) pancreatectomized. In brief, rats were fasted overnight and anesthetized using ketamine-HCl (60 mg/kg), pentobarbital-Na (20 mg/kg) and almost entire splenic portion of the pancreas was removed. After 48 h, partially pancreatectomized rats were sacrificed by cervical dislocation and immediately dissected to remove the regenerating pancreas. The excised tissues were placed in chilled phosphate buffered saline containing a protease inhibitor complex and homogenized. Homogenates were centrifuged at 3,000 rpm for 10 min at 4°C and then at 12,000 rpm for 20 min at 4°C. The final clear supernatant was analyzed for the protein content using Bradford's method.

Extracts were then stored as aliquots at -70°C until further use.

Viability test

At day 17 of culture, the differentiated clusters were examined for viability by trypan blue exclusion. The insulin content of the cell clusters was evaluated by staining with diphenyl thiocarbazone (DTZ). Stock solution was prepared as previously described [17] by dissolving 50 mg of DTZ (Sigma) in 5 ml of dimethyl sulfoxide (DMSO) and was stored at -20°C. During staining 10 µL of stock solution was added to 1 ml of culture medium. Differentiated and undifferentiated cells were incubated at 37°C for 30 minutes in DTZ containing medium. After the plates were rinsed three times with Hanks balanced salt solution, cells were examined with stereomicroscope.

Determination of insulin secretion

As we previously described [14], 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 (BSA). This was followed by incubation in KRB containing 5.5, 12, or 25 mM glucose concentration for additional 2 hours. The KRB was collected and frozen at -70°C until assayed. Insulin assay was performed by enzyme-immunoassay (Linco Research Inc. Missouri, USA) according to the manufacture's instruction.

Determination of intracellular insulin and C-peptide content

The formed clusters were washed 3 times with PBS. They were then suspended and dispersed in 50 Mm HCL/70% ethanol. After centrifugation at 8000 rpm for 5 minutes the supernatant was collected from the cell lysate and neutralized by the addition of 50 Mm NaOH. Insulin and C-peptide concentrations in the supernatants were determined by rat insulin and c-peptide ELISA Kits as described before [14].

Mass-balance of the insulin-releasing cell cluster

A mass-balance for sequestered insulin was also calculated by comparison of the quantity of insulin in the cell clusters fully equilibrated with media and the quantity of insulin released during glucose stimulation [18]. The volume of a single cluster was measured, based on the assumption that the cluster was hemi ellipsoidal in the shape (volume = $3/4\ \pi\ r_1r_2r_3$) with major and minor axes, obtained from optical microscopy.

Flow cytometric verification of rat insulin production

As we described before [15], clusters were collected by centrifugation and re-suspended in 0.5-1.0 ml PBS (8 g Nacl, 0.2 g Kcl, 1.44 g Na_2HPo4 and 0.24 g KH_2PO4 in 1000 dH2o, pH 7.4). Formaldehyde was added to final concentration of 2-4% formaldehyde. Cells were fixed for 10 minutes at 37°C and chilled on ice for 1 minute. Cells were permeabilized by adding ice-cold 100% methanol slowly to pre-chilled cells to final concentration 99% methanol. Cells were incubated 30 minutes on ice. After centrifugation, 2-3 ml of incubation buffer (0.5 g bovine serum albumin in 100 ml PBS) was added and rinsed by centrifugation. Cells were re-suspended in 100 µl incubation buffer and incubated for 10 minutes at room temperature. The primary antibody; monoclonal anti-rat insulin (cell Signaling technology) was added and incubated for 30-60 minutes at room temperature. The cells were then rinsed as before in the incubation buffer by centrifugation. Cells were re-suspended in fluorochrome-conjugated secondary antibody; fluoresce in isothiocyanate labeled polyclonal swine anti-rabbit immunoglobulin/FITC 1:10 (Dako cytomatin Denmark), and then were incubated for 30 minutes at room temperature. The cells were rinsed as before and resuspended in 0.5 ml PBS. The labeled cells were then analyzed using argon ion laser 15 mw with a wave length of 488 nm (FACS Calibur, Becton-Dickinson, USA). A total of 10000 events were obtained and analyzed with the cell Quest software program (Becton-Dickinson, USA). Rat pancreatic islets served as a positive control.

Detection of islet related gene expression

Gene expression levels of insulin (INS), glucagon (GCG), somatostatin (SST), pancreatic polypeptide (PPY), pancreatic and duodenal homeobox1 (PDX-1), neurogenin 3 (Ngn-3), neurogenic differentiation 1 (NeuroD1) and paired box gene (PAX-6) were determined by Reverse Transcription

Polymerase Chain Reaction (RT-PCR). Glyceraldehayde-3-phosphate dehydrogenase (GAPDH) was included as an internal control. The primer sequences for RT-PCR are shown in Table 1. Briefly, total RNA was extracted from adult rat pancreas, as well as from untreated and treated bone marrow cells with TRIzol reagent according to manufacturer's instructions (Invitrogen Corporation Grand Island, NY, USA). Reverse transcriptions were carried out using 1µg of total RNA and cDNA kit (High Capacity cDNA Archive Kit, ABI Prism, CA, USA). 2 µl of cDNA sample were mixed with 1 µl of each primer and 10 µl of 2 X Taq PCR master mixes (Qiagen, CA, USA). Distilled water was added to a volume of 20 µl and the resulting mixture was subjected to PCR thermal cycler. The cycling parameters were as follows: initial denaturation at 95^{o}C for 5 minutes, followed by 30 cycles of 94^{o}C for 30 seconds, annealing at 53^{o}C - 57^{o}C (depending on the primers) for 30 seconds, elongation at 72^{o}C for 30 seconds and the final extension at 72^{o}C for 10 minutes. The resulting products were electrophoresed in 1% agarose gel to detect gene bands and photographed by canon digital camera.

Quantitative RT-PCR assay (qPCR)

To clarify the *in vitro* expression levels of PDX-1, insulin, glucagon and somatostatin (SST) genes of the HSCs after induction, fluorescent quantitative RT-PCR was performed. Rat islets served as a positive control. The expression levels of genes were analyzed with a real time PCR instrument (ABI 7000 PRISM, Applied Biosystem, USA). Amplifications were done in 25 µl total reaction volume containing 12.5 µl 2X SYBR Green Master Mix (Fermentas), 1 µl of cDNA template and 25 p mole of each primer. GAPDH gene was used as an endogenous control. Each gene was tested with three independent replications. The results were calculated by mathematical model of relative quantifications [19].

Immunohistochemical studies

For immunohistochemical analysis, differentiated clusters were processed to paraffin blocks. Sections were dewaxed in xylene and rehydrated in descending grades of alcohol (Ethanol anhydrous denatured, histological grade 100% & 95%). Microwave antigen retrieval in 10mM sodium citrate at pH 6 was carried out for 20 minutes. Sections were subsequently covered with 3% hydrogen peroxide for 10 minutes. After sections were covered by blocking solution for 1 hour, the diluted primary antibody was applied to slides. Primary antibody concentrations were as follows: 1/100 monoclonal rabbit anti rat insulin (Cell Signaling Technology) and 1/100 rabbit anti rat C-peptide (Cell signaling Technology). Primary antibodies were incubated overnight at 4°C followed by addition of diluted secondary antibody, polyclonal swine anti rabbit immunoglobulin FITC (DakoCytomation). Avidin-Biotin complex reagent was applied to slides for 30 minutes in room temperature. For visualization, 3, 3' diaminobenzidine tetrahydrochloride (DAB) reagent was used to cover the slides for 5 minutes. Hematoxylin was used as a counter stain.

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

Genes	Forward primer	Reverse primer	An.temp (°C)	(bp)
Insulin1	CCTGCCCAGGCTTTTGTCA	GGTGCAGCACTGATCCACAATG	57	231
Glucagon	ATCATTCCCAGCTTCCCAGA	CGGTTCCTCTTGGTGTTCAT	54	162
Somatostatin	CAGGAACTGGCCAAGTAC	AGTTCTTGCAGCCAGCTTTG	54	187
p.polypeptide	CGCATACTACTGCCTCTCCC	CAGCAGCGCAGGGCATCAAA	53	278
PDX-1	GGTGCCAGAGTTCAGTGCTAA	CCAGTCTCGGTTCCATTCG	53	249
Ngn-3	CTGCGCATAGCGGACCACAG	CTTCACAAGAAGTCTGAGAA	57	233
NeuroD1	TGTCGTTACTGCCTTTGGAA	CGATCTGAATACAGCTACACGAA	51	153
PAX-6	CGACAAGATTTGCCATGGAT	CAACCTTTGGAAAAACCAACA	51	179
GAPDH	CACCCTGTTGCTGTAGCCATATTC	GACATCAAGAAGGTGGTGAAGCAG	57	196

Finally, sections were dehydrated, cover-slipped and examined. Positive results were identified as a brown color at the antigen site.

Immunofluorescence staining

The cryospinned islet-like clusters were frozen sectioned at 8 µm, fixed with 2-4% formaldehyde in PBS for 15 minutes at room temperature and rinsed with PBS for three times, 5 minutes each. The cells were then incubated with ice-cold 100% methanol for 10 minutes followed by blocking the specimen in 5% of rat normal serum in PBS/Triton (BDH Laboratory Supplies Poole, England), for 60 minutes. The cells were incubated overnight at 4°C with the following primary antibodies; rabbit monoclonal anti-insulin 1:200 (Cell Signaling Technology, Danvers, MA, USA) and rabbit polyclonal anti-c-peptide 1:100 (Cell Signaling Technology). Subsequently, the cells were incubated with the secondary antibody; polyclonal swine anti-rabbit immunoglobulin labeled with FITC (DakoCytomation, Glostrup, Denmark) at 22°C for 2 hours. The nuclei were counter-stained with DAPI (Invitrogen, UK). The negative controls were obtained by eliminating the step of the overnight incubation with the primary antibodies. Specimens were immediately examined and photographed using a fluorescence microscope and the blue filter (Olympus, Japan).

Statistical analysis

Data were represented as Mean ± S.E.M (standard error of means) statistically significant differences and analysis of variance were calculated using ANOVA test (SPSS for Windows, 15.0 version). Statistical significance was set at $P < 0.001$.

RESULTS

Flow cytometric analysis of isolated HSCs

Flow cytometric analysis of the isolated non-adherent bone marrow stem cells revealed that these cells were negative for CD44, CD29 and CD106 and they expressed low levels of CD34 (Figure 1). These results indicated that they were not mesenchymal stem cells.

Differentiation of HSCs into islet like clusters

Hematopoietic rich stem cells obtained and cultured as described underwent a series of changes as demonstrated in (Figure 2A-B). During differentiation, the cells gathered gradually in groups with formation of cell clusters. At the end, cell aggregates that mimic islet architecture became compact and formed islet-like clusters. The number of clusters formed on each cover slip was 500 ± 45.

Viability testing and functional evaluation of the differentiated clusters

We examined the clusters formed in culture (Day 17) for cell viability by trypan blue-exclusion. The assay revealed viable clusters. Also to evaluate the insulin-producing cells in cultures, we stained the differentiated cells with zinc-cheliating agent (DTZ). DTZ is zinc-binding substance, and

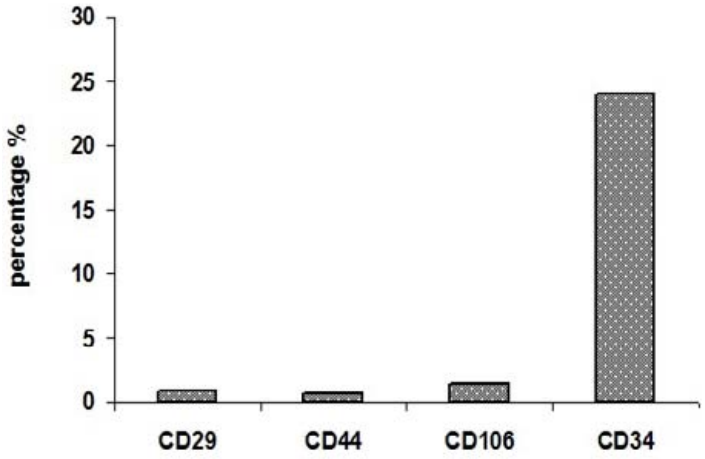


Figure 1. Surface marker analysis of undifferentiated stem cells by flowcytometry.

pancreatic islets are known to stain crimson red after DTZ treatment. Differentiated cells in the islet-like clusters were distinctly stained crimson red by DTZ (Figure 3), while undifferentiated cells were not.

Insulin release and c-peptide content in the differentiated cells

Differentiated cell clusters released increasing amounts of insulin in a glucose-concentration-dependent fashion (Figure 4). The mean insulin secretion by 100 clusters was 0.8 ± 0.04 ng/mL (1.4 ± 0.04 ng/mg protein/hour) in response to 5.5 mM glucose, 3 ± 0.3 ng/mL (5 ± 0.3 ng/mg protein/hour) in response to 12 mM glucose and 5 ± 0.6 ng/mL (8.5 ± 0.6 ng/mg protein/hour) when the 25 mM concentration was used. These results represent the mean of 6 experiments; differences between these readings were statistically significant ($P < 0.001$). When the glucose concentration was 25 mM, the content in the same number of clusters ranged between 255 and 323 ng/mg protein for insulin and 1.6 - 1.8 ng/mg protein for C-peptide.

These data indicated that, there was a stepwise increase in insulin release by the formed cell clusters in response to increasing glucose concentrations.

Mass balance around insulin-releasing cell cluster

Table 2 contains the details of a mass-balance calculation for sequestered insulin. Assuming Fickian diffusion, the quantity of insulin in cell clusters fully equilibrated with media can readily be calculated as the product of the volume of a single cluster and the maximum concentration of insulin in the media to which the cells are exposed. The volume of a single cluster was 1.4×10^{6} μm^3 (1.4×10^{-6} ml) with major and minor axes ($r_1 = 150$ µm, $r_2 = 75$ µm, $r_3 = 60$ µm) obtained from optical microscopy. The total volume of 100 clusters that were used in glucose stimulation was 14×10^{7} μm^3 (14×10^{-5} ml). At 100% equilibration with an insulin concentration of 7.5 ng/ml, 100 clusters would contain 0.001 ng of insulin. However, the quantity of insulin released during glucose stimulation by 100 clusters was 5 ng, much greater than they would contain if fully equilibrated with the highest concentration of insulin to which they had been exposed.

Detection of insulin by flow cytometry

Moreover, verification of insulin produced by the formed clusters was revealed by flow cytometric analysis (Figure 5). About 16% of the cells were positive for insulin.

Gene expression by RT-PCR in differentiated islet-like clusters

Pancreatic development and gene expression are regulated by specific transcription factors. To determine whether endocrine-specific transcription factors and pancreas specific genes were expressed in the differentiated islet-like clusters, PCR analysis was performed. The PCR product for insulin 1, glucagon, somatostatin and pancreatic polypeptide genes were visible in differentiated clusters and were not detected in undifferentiated hematopoietic stem cells. Transcripts for PDX-l, neurogenin-3, NeuroD1, and PAX-6 were not detectable in undifferentiated bone marrow hematopoietic stem cells; in contrast, they were up-regulated in differentiated clusters (Figure 6). Gene expression analysis in differentiated islet like clusters was similar to that in rat pancreas tissue and confirmed the differentiation of bone marrow hematopoietic stem cells into islet-like clusters *in vitro*.

Quantitative PCR

Relative quantitative PCR was performed to determine the expression profile of differentiated HSCs. QPCR analysis showed that there was an exponential-like increase in the expression of endocrine hormones and transcription factor (Figure 7). The expression levels of INS, SST, GCG and PDX-1 in undifferentiated cells were virtually undetected, while differentiated clusters were 0.24, 0.29, 0.013 and 0.18 fold respectively comparing to rat islet genes.

Immunocytochemical and Immunofluoresence studies

Both Immunocytochemical and Immuno-fluorescence staining of the differentiated cell clusters were positive for insulin and c-peptide (Figure 8 and 9).

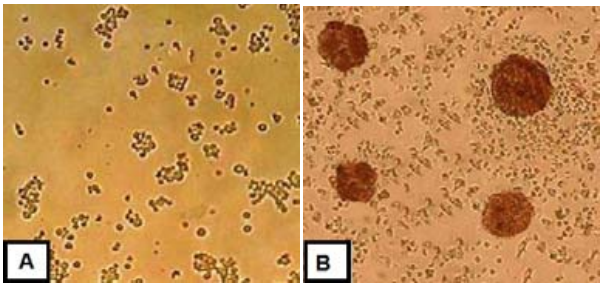


Figure 2. Morphological changes of HSCs during differentiation. **(A)** Undifferentiated HSCs, one day after isolation (X60). **(B)** Well formed clusters with spheroid configuration after 17 days of differentiation (x60).

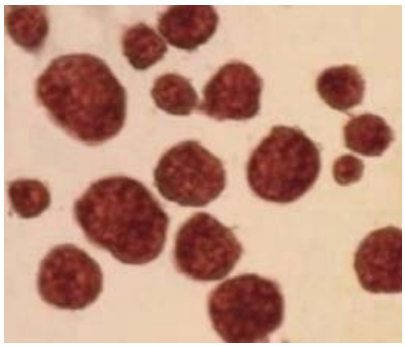


Figure 3. Ditizone staining of islet-like clusters. The clusters distinctly stained crimson red by DTZ (X 200).

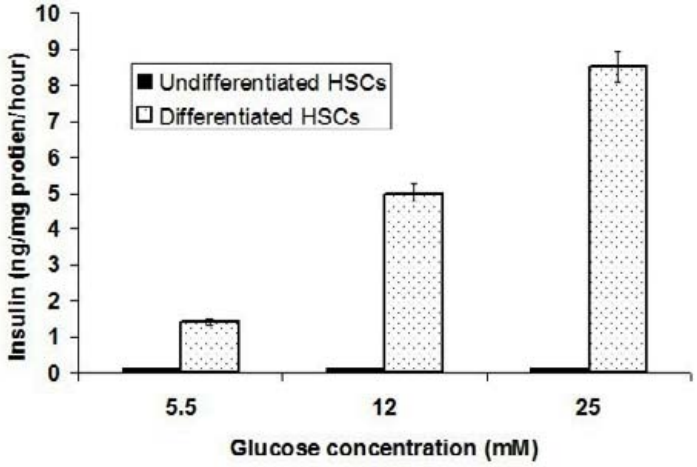
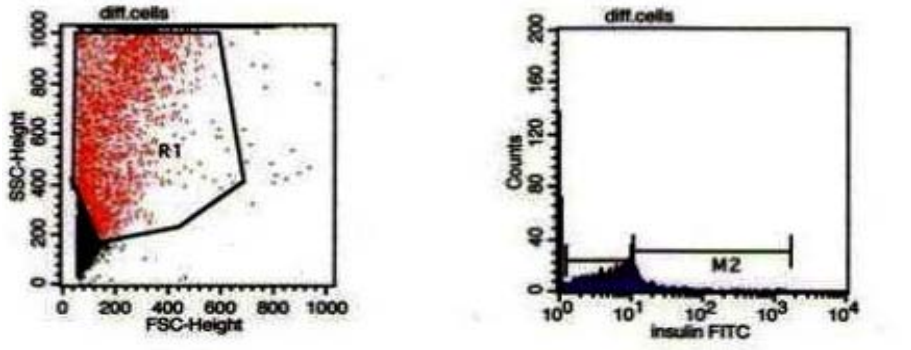


Figure 4. Insulin release in response to glucose stimulation as detected by immunosorbent assay. Clusters treated with 25 mM glucose secreted insulin nearly 6 times higher than that when treated with 5.5 mM glucose.



Marker	Left, Right	Events	% Gated	CV	Median	Peak Ch
All	1, 9910	2576	100.00	492.07	5.73	1
M1	1, 10	1904	73.91	51.01	4.74	8
M2	11, 1700	423	16.42	323.31	13.46	11

Figure 5. Verification of insulin secretion by flow cytometric analysis showed 16.4% positive cells.

Table 2. Mass balance around differentiated clusters.

	Input		Output	
Volume	Volume of 100 clusters	$14x10^{-5}$ /ml	Volume of supernatant	1 ml
Concentration	Insulin in culture media	7.5 ng/ml	Insulin in supernatant after stimulation	5 ng/ml
Quantity	Insulin uptake at 100% equilibrium	0.001 ng/ml	Quantity released	5 ng/ml

The uptake of insulin into the clusters at complete equilibrium is the product of clusters volume and insulin concentration in culture medium. The amount of insulin released during glucose stimulation is the product of concentration of insulin and volume of supernatant.

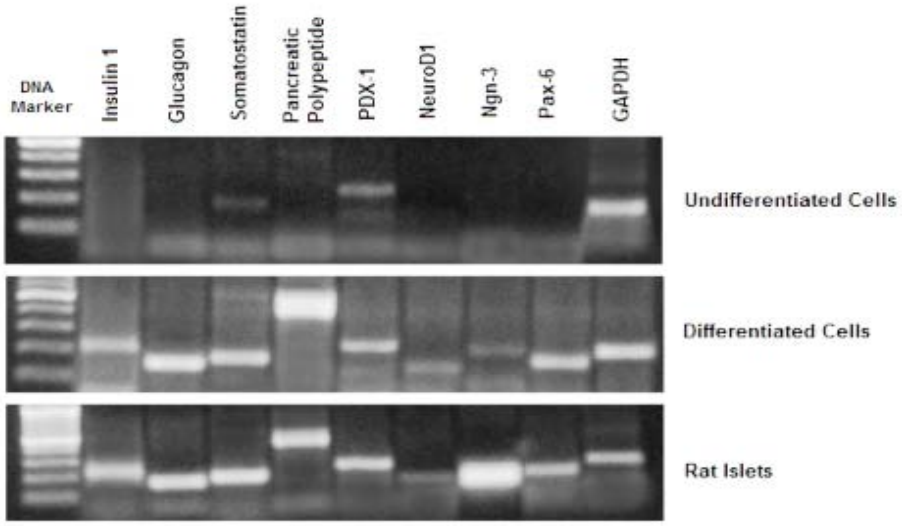


Figure 6. Gene expression of undifferentiated HSCs, islet-like clusters and rat pancreas (control). Clusters as well as rat pancreas expressed similar endocrine genes and transcription factors.

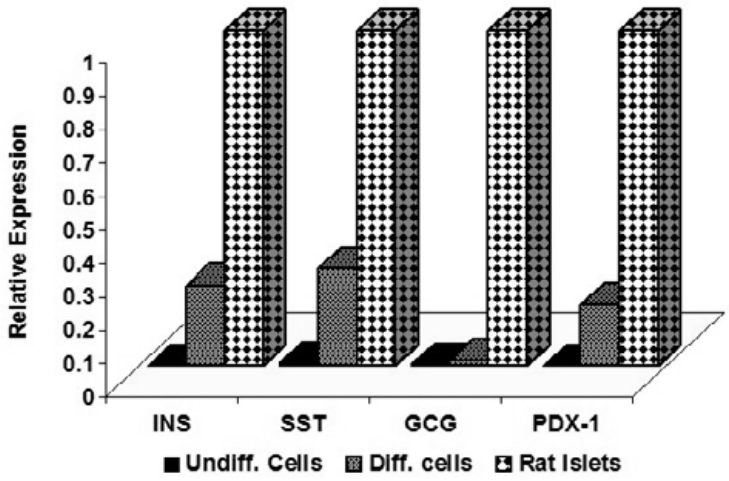


Figure 7. Quantitative PCR analysis for INS, SST, GCG and PDX-1 genes. Insulin gene expression was about 5% of that in rat islets.

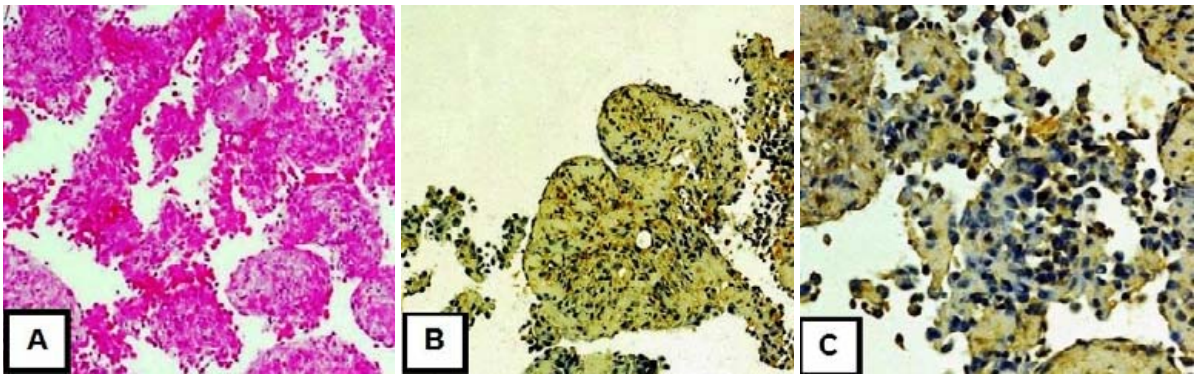


Figure 8. Immunocytochemical staining of hematopoietic stem cells for insulin and c-peptide. **(A)** Hematoxylin and eosin staining of the differentiated clusters (X100). **(B)** Differentiated cells (positive for insulin, x 100). **(C)** Differentiated cells (positive for c-peptide, x 100).

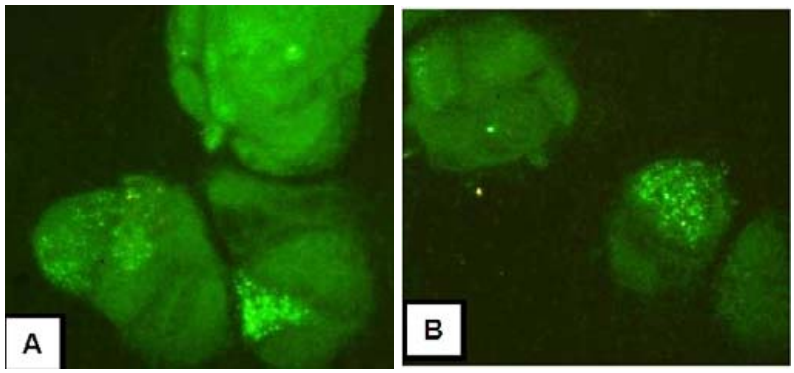


Figure 9. Immunofluorscence staining of hematopoietic stem cells for insulin and c- peptide. **(A)** Differentiated cells (positive for insulin, x 200). **(B)** Differentiated cells (positive for c-peptide, x 200).

DISCUSSION

Recent study has demonstrated the presence of progenitor cells within pancreatic islets that are capable of differentiating into insulin producing cells [20]. Other reports have stated hepatic oval cells [6] and intestinal epithelium [21] as well as the pluripotent embryonic stem cells [22] were capable of differentiating into cells with a pancreatic endocrine phenotype. However, even with the progress occurred by these findings, some obstacles, such as immune rejection and autoimmunity against newly formed β-cells, still remain. To overcome these limitations, we explored the possibility of using bone marrow derived stem cells as sources for trans-differentiation into insulin-producing cells under specific *in vitro* culture condition. Bone marrow has been known for years to represent a safe and abundant source for large quantities of adult stem cells. Hematopoietic stem cells are normally present in adult marrow and have been shown to differentiate into a variety of cell types, including bone, muscle, fat, cartilage, cardiomyocytes and hepatic cells [23-27]. In this study, we isolated, trans-differentiated and characterized hematopoietic bone marrow cells into insulin producing cells.

The isolated stem cells were negative for CD44, CD29 and CD106 and slightly positive for CD34, which indicates that they were not mesenchymal stem cells. There are many differences between the human and mice hematopoietic cell markers

for the commonly accepted type of hematopoietic stem cells, where expression of CD34 is low or negative in mouse but positive in human [28].

The mammalian pancreas arises initially as dorsal and ventral buds that emanate from the embryonic foregut endodermal layer and differentiates into the endocrine cells forming the pancreatic islets of Langerhans under a cascade of gene activation events controlled by transcription factors, including PDX-l, Ngn-3; PAX-6 [29, 30]. Inducing stem cell to differentiate into islet-like cells resembles this reprogrammed process.

Several *in vitro* studies have shown that bone marrow-derived cells are capable of being reprogrammed to become functional insulin-producing cells [20, 31, and 32]. Their inducing processes are to initiate PDX-l gene expression using factors such as β-mercaptoethanol, dimethyl sulphoxide, trichostatin A. We attempted to induce hematopoietic cells into islet-like cells by dimethyl sulphoxide, high glucose, nicotinamide and exendin-4 which were considered as potent inducers for pancreatic islet differentiation. Glucose is not only a growth factor for β-cell replication *in vitro* and *in vivo* at a 20-30 mmol/l concentration, but also induces adult hepatic stem cells into pancreatic endocrine hormone-producing cells at a 23 mmol/l concentration and increase insulin content in cell lines derived from embryonic stem cells at a 5 mmol/l concentration [33, 34, 35]. Nicotinamide is a poly (ADP-ribose) synthetase inhibitor and could induce pancreatic progenitor cells into insulin producing cells [7]. Whereas exendin-4 could also stimulate both β-cell replication and neogenesis from ductal progenitor cells and inhibit apoptosis of β-cell [36].

Insulin production was confirmed by staining with zinc chelating agent (DTZ). Furthermore, their gene expression was similar to that of pancreatic endocrine tissue. These cells were not only capable of insulin production but also of its release in a dose-dependent fashion according to the glucose concentration. The expression of insulin, glucagon and somatostatin genes could be demonstrated in the differentiated clusters. Evidence has also been provided that PDX-l, Ngn-3 and NeuroD1 were up regulated. The qPCR is consistent with RT-PcR suggesting that insulin gene expression in the cellular fraction was about 5% of that in rat islets. Some investigators claimed that, part of insulin detected may have been derived from insulin added to the culture media in certain protocols or insulin present in serum. The stepwise increase in the insulin release as a function of the glucose concentration does not support these assumptions. In addition; insulin was detected by flow cytometry as we proved in Figure 5. Moreover, our results using immunocytological (Figure 8) and immunofluoresence detection of c-peptide (Figure 9) and also insulin mass balance (Table 2) confirmed that insulin released was the result of endogenous synthesis. Recent studies support this conclusion; a group of Chinese investigators [37] reported their success in the differentiation of bone marrow mesenchymal stem cells from a diabetic patient into functional insulin producing cells *in vitro*.

Their results showed that, the use of the diabetic patient's own bone marrow stem cells as a source of autologous insulin producing cells for β-cell replacement could be feasible.

In conclusion, our findings present evidence that BM may include a pancreatic progenitor cell capable of differentiating into functioning endocrine hormone-producing cells. The differentiated cells produce insulin in response to glucose challenge. Immunofluoresence and Immunohistochemical studies showed positive staining of insulin and c-peptide. Also flow cytometric analysis and expression of endocrine genes proved the production of insulin. Several problems have to be solved before this becomes a clinical reality. Establishment of a reproducible protocol is one. Large number of insulin producing clusters produced *in vitro* need to be obtained for transplantation. Several growth or differentiating factors should be tested and better culture conditions have to be investigated. The utilization of an extra cellular matrix may be important. The possible advantages of bioreactors should also be explored. The cell product has to mimic some phenotypic traits of the mature β-cell, such as glucose sensing, insulin processing and secretion

in appropriate amounts. The expression of auto antigens has to be abrogated in order to avoid the regain of autoimmune response. Lastly, the conditions of the site for transplantation of these cells must be optimized.

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